Characterization of the *nod* and *sdh* operons in the legume symbionts *Bradyrhizobium japonicum* and *Sinorhizobium meliloti.*

by

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"Great things are not done by impulse,
but by a series of small things brought together."

- Vincent van Gogh

Abstract

Ph. D. **Frédéric D'Aoust** **Microbiology**

Characterization of the *nod* and *sdh* operons in the legume symbionts *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*.

This study was undertaken to characterize the *nod* and *sdh* operons of *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*. Ten putative *B. japonicum* mutants with altered *nod* gene induction characteristics were isolated by screening mutants for genistein-independent *nod* gene expression. The mutants were found to have higher *nodY* expression than the wild-type in the presence of genistein. The increased sensitivity of all mutants to genistein was more apparent under suboptimal inducer concentration (0.1μM) and/or temperature (15°C). The expression of *nodY* gene induction was determined for five strains (Bj30050, 53, 56, 57, 58) under different temperature and inducer conditions. These five strains were also found to produce more lipochitooligosaccharide than the wild-type, at both 25°C and 15°C. Three of the ten mutant strains (including Bj30056 and 57) were unable to fix nitrogen with soybeans grown at optimal temperatures. Based on *nodY* gene expression and symbiotic phenotype the *B. japonicum* mutants were classified into three groups.

A molecular genetic approach was taken to investigate the regulation of expression of succinate dehydrogenase (SDH) in *S. meliloti*. The *sdhCDAB* genes encoding SDH were shown by RT-PCR to be co-transcribed and thus constitute an

operon. The transcriptional start site and putative promoter region of the first gene in the operon, *sdhC*, were identified by 5'-RACE and DNA sequence analysis. Transcriptional *lacZ* fusions to *sdhC* indicated that expression of the operon is regulated by carbon source in the growth medium but not by growth phase. The highest expression of the *sdh* operon was observed in cells grown with acetate, arabinose and glutamate, as sole carbon sources, and the lowest expression was observed in cells grown with glucose and pyruvate as sole carbon sources.

Also presented is the isolation and characterization of the first defined *sdh* mutant in a rhizobial species. The mutants helped demonstrate that the total lack of SDH activity would be lethal to *S. meliloti* cells. Symbiotic phenotype of the mutants indicated that SDH is required for N_2-fixation.

Résumé

Ph. D. **Frédéric D'Aoust** **Microbiologie**

Charactérization des opérons *nod* et *sdh* des symbionts de légumineuse *Bradyrhizobium japonicum* et *Sinorhizobium meliloti.*

Cette étude avait pour but de charactériser les opérons *nod* et *sdh* de *B. japonicum* et de *S. meliloti*. Dix mutants de *B. japonicum* altérés au niveau de l'induction du gène *nod*, ont été isolés par criblage pour leur capacité à exprimer leur gène *nod* indépendemment de la génisteine. L'expression du gène *nodY* était plus élevée chez les mutants en présence de génisteine. La plus grande sensibilité de tous les mutants était plus évidente en condition de concentration d'inducteur et de température sous optimales (0.1 mM et/ou 15°C). L'expression du gène *nodY* a été déterminée en différente conditions d'inducteur et de température chez cinq souches mutantes (Bj30050, 53, 56, 57, 58). Ces mutants produisaient plus de lipo-chito-oligosaccharides (LCO) que la souche mère à 25°C et 15°C. Trois des dix souches mutantes (incluant Bj30056 et 57) ne pouvaient pas fixer l'azote chez le soya cultivé à température optimale. Les mutants de *B. japonicum* ont été classifiés en trois groupes, sur la base de l'expression de leur gène *nodY* et de leur phénotype symbiotique.

Une approche de génétique moléculaire a été appliqué afin de découvrir la régulation et le rôle du complexe enzymatique de la succinate déhydrogénase dans la fixation symbiotique de l'azote dans la bactérie *S. meliloti*. Les gènes codant le SDH

ont été isolés et leurs organisation en opéron a été découverte. Le site de démarrage transcriptionel et la région promotrice ont été déterminés par analyse 5'-RACE. Les fusions transcriptionelles de *lacZ* à la région promotrice du *sdh* ont indiqué que l'expression de l'opéron était catabolique et dissociée de la phase de croissance. Les plus hauts taux d'expressions de l'opéron *sdh* ont été observés lorsque l'acétate, l'arabinose et le glutamate agissaient comme unique source de carbone; les plus bas taux ont été observés en présence de glucose et de pyruvate.

Le premier mutant *sdh* caractérisé chez les rhizobia a été isolés et caractérisé. L'analyse des mutants a démontré que l'absence totale de l'activité de SDH est fatale à la survie de *S. meliloti*. Le phenotype symbiotique des mutants a indiqué que SDH est requise pour la fixation de l'azote atmospherique.

Contributions to knowledge

1. Ten *B. japonicum* mutants (Bj30050 to Bj30059) with increased sensitivity to genistein were isolated and characterized. The mutants were grouped into three classes based on *nod* gene expression and symbiotic phenotype.

2. Demonstrated that the *B. japonicum* mutants with increased sensitivity to genistein were able to partially overcome low root zone temperature (RZT) inhibition of nodulation of soybean plants. This further supported the notion that reduced sensitivity to the plant signal is the main mechanism responsible for inhibition of nodulation observed at low RZT.

3. Mutants with increased sensitivity to genistein were shown to have increased LCO production under both optimal and suboptimal RZT compared to wild-type strains.

4. Determined that the *sdhCDAB* genes that encode SDH in *S. meliloti* are an operon which is cotranscribed as a single polycistronic mRNA from a single promoter found upstream of *sdhC*.

5. Determined by 5'-RACE analysis that the *sdhCDAB* transcript is initiated from a transcriptional start site 84 bp upstream of *sdhC*. Putative -35 and -10 promoter

regions were identified and shown to have some similarities with other previously reported bacterial *sdh* promoters.

6. Demonstrated that expression of the *sdh* operon is regulated by carbon source in the growth medium but not by growth phase, by use of transcriptional *sdh*::lacZ gene fusions.

7. Isolated the first genetically well-defined *S. meliloti* SDH mutants. Three mutants (Rm300168, Rm30069, Rm30070) unable to use succinate as sole carbon source were isolated, and the mutations were mapped to the anchor subunits: two in *sdhC* (*sdhC172*::EZ::TN; *sdhC80*::EZ::TN) and one in *sdhD* (*sdhD280*::EZ::TN).

8. Demonstrated the effective use of EZ::TN mutagenesis for the isolation of mutants defective in genes, such as those in operons, that lead to lethal polar effects on downstream genes.

9. Isolated the wild-type *S. meliloti sdh* operon by complementation of the *sdh* mutants strains with a cosmid clone bank.

10. Demonstrated by symbiotic phenotype analysis that SDH is required for symbiotic N_2-fixation.

11. Demonstrated that SdhC and SdhD are required for proper anchoring of the SDH enzyme to the membrane complex in *S. meliloti*. SdhA and SdhB were shown to be responsible for the enzyme activity and required for growth of *S. meliloti*.

12. Demonstrated that SDH expression is induced by TCA cycle intermediates.

13. Isolated and characterized three non-*Bradyrhizobium japonicum* endophytic bacteria with plant-growth-promoting capabilities. The strains were identified as *Bacillus subtilis* (NEB4, NEB5) and *Bacillus thuriengiensis* (NEB17) strains.

List of abbreviations

AcN	acetonitrile
ACN	aconitase
Amp	ampicillin
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BNF	biological nitrogen fixation
BUGM	Biolog universal growth medium
CIS	citrate synthase
Cm	chloramphenicol
CoA	coenzyme A
DEPC	diethyl pyrocarbonate
DIG	Digoxigenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FDR	fumarate reductase
FUM	fumarase
Gm	gentamicin
HPLC	high pressure liquid chromatography
HV	hypervariant
ICD	isocitrate dehydrogenase
IncP	incompatibility group
IPTG	isopropyl-β-D-galactyopyranoside
Km	kanamycin
LB	Luria-Bertani medium
LBmc	Luria-Bertani medium with magnesium and calcium
LCO	lipo-chito-oligosaccharide
LSD	least significance difference
M9	minimal medium
MDH	malate dehydrogenase
MU	Miller units
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NBT	nitro blue tetrazolium chloride
NEB	non-*Bradyrhizobium* endophytic bacteria
Nm	neomycin
NTG	*N*-methyl-N'-nitro-*N*-nitrosoguanidine
OD	optical density
OGD	2-oxoglutarate dehydrogenase
ONPG	o-nitrophenyl-β-galactopyranoside
PCK	phosphoenolpyruvate carboxykinase

PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PGPB	plant-growth-promoting bacteria
PGPR	plant-growth-promoting rhizobacteria
PHB	poly-β-hydroxybutyrate
Pi	inorganic phosphate
PYC	pyruvate carboxylase
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
RZT	root zone temperature
SCS	succinyl-CoA synthetase
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulphate
SDW	shoot dry weight
SIM	similarity index
Sm	streptomycin
Sp	spectinomycin
TAE	tris-acetate-EDTA buffer
Tc	tetracycline
TCA	tricarboxylic acid cycle
TY	tryptone yeast medium
UV	ultra-violet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YM	yeast mannitol medium
YS	yeast salts medium

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Contributions of authors

All of the experiments described in this thesis were designed and performed by myself, under the guidance of my supervisor Dr. B.T. Driscoll with the exception of the contributions described below.

Chapter 3 was drawn from a published article co-authored by myself along with Hermia Ip, Anjuman A. Begum, Hao Zhang, Don L. Smith, Brian Driscoll, and Trevor C. Charles. Hermia Ip was a M.Sc. student at the Biology Department, University of Waterloo, under the supervision of Dr. Trevor Charles and provided assistance with characterization of *B. japonicum* mutants using β-galactosidase assays. She was especially vital in determining the *nod* gene kinetics of the mutant strains (data not presented in this thesis) as the β-galactosidase assays were performed in parallel experiments between our two labs, resulting in similar results. Hao Zhang was a Ph.D. student in the Department of Plant Science (McGill University) under the supervision of Dr. Don Smith and provided technical assistance in determining the LCO production of *B. japonicum* mutants. Anjuman A. Begum was a post-doctoral researcher in our laboratory and was responsible for optimization of the UV mutagenesis protocol as well as providing help with the initial isolation of the *B. japonicum* mutants. The published manuscript was originally written by the different authors and subsequently edited and revised by Dr. Brian Driscoll and Dr.Trevor Charles.

Appendix 1 was drawn from a publication co-authored by myself in collaboration with Yuming Bai, Don Smith, and Brian Driscoll. Yuming Bai was a Ph.D. student from Department of Plant Science (McGill University) under the supervision of Dr. Don Smith and was responsible for the initial isolation of the NEB

strains. Dr. Bai was responsible for the growth pouch experiments. The published manuscript was originally written by myself and subsequently edited and revised by Dr. Brian Driscoll, with contributions from the other authors.

Chapter 1. Literature review

Introduction

While molecular dinitrogen (N_2) is the most abundant component (approximately 80%) of the earth's atmosphere, most organisms are unable to directly use it for growth and reproduction. Before it can be used by a living organism, N_2 must first be reduced either chemically or biologically to NH_4^+ by a process commonly called N_2-fixation. Because fixed N is continuously being depleted by microbial denitrification, soil erosion, chemical volatilization, leaching, and crop harvesting, it is often considered to be a growth-limiting factor for plants in most soils (Bloom, 1997). For continuous crop production, it therefore becomes imperative to continually replenish the available N in soil.

Currently, in developed countries, the most widely employed method to replenish soil N is via the use of commercial inorganic N fertilizers. But before the advent of modern N fertilizers, farmers relied on biological nitrogen fixation (BNF) to renew the N content of the soil. With the cost of fossil fuels reaching record highs and pressure by various groups for environmental sustainability, there is renewed interest BNF. To our knowledge, BNF is limited to a large and diverse group of prokaryotes, including both eubacteria and archaea (Zehr *et al.*, 2003), with the best characterized and studied BNF system being the rhizobia-legume symbiosis.

The increased importance of legume crops such as soybeans and alfalfa in world agriculture has helped stimulate research in the field of symbiotic N_2-fixation.

Soybeans are one of the most important crops cultivated in Canada with an annual production of approximately 1 000 000 ha. The ability of this legume to fix N_2 in specialized root modules has helped propel it to the third most important crop in Quebec (0.34 Mt soybean production in 1997-98). During symbiotic association with *Bradyrhizobium japonicum,* soybean plants can fix up to 200 kg·N/ha/yr (Smith and Hume, 1987), eliminating the need for expensive N fertilizers that can become detrimental to the environment. To put this in perspective, by growing soybeans instead of other crops, Canadian producers save an estimated 100 kg/ha of N, equivalent to 105,000 t of N fertilizer, or 77 million dollars (Agricultural and Agri-Food Canada, 1999).

The potential benefits of N_2-fixation have been known for quite some time. As early as the third century BC, Theophrastus reported on enrichment of soil with the use of faba beans (as cited in Raven *et al.*, 1996). In 1890, Beijerink demonstrated the nodulation of sterile bean plants by nodule-isolated pure cultures (Broughton and Perret, 1999). Since then our knowledge of the process involved in N_2-fixation has greatly increased.

Rhizobia-Legumes: symbiotic partners

Members of the family *Rhizobiaceae* are characterized by their ability to enter into a N_2-fixing symbiotic relationship with plants, where their role is to fix atmospheric dinitrogen in specialized structures called nodules. This association seems to be beneficial to both the bacteria and the plant. Protected within the nodule, the bacteria receives a steady supply of photosynthetic by-products from the

plant in the form of C_4-dicarboxylic acids. In exchange the bacteria reduces N_2 into NH_4^+ which it transfers to the plant host, greatly increasing its competitive advantage under N-limiting conditions (De Philip *et al.*, 1992).

Most of the species capable of symbiotic N_2-fixation belong to the α-proteobacteria order *Rhizobiales*, including the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium.* Recent evidence, however, suggests that some β-proteobacteria, such as *Burkholderia* and *Ralstonia* may also establish similar interactions (Chen *et al.*, 2003; Sawada *et al.*, 2003). When not participating in symbiosis, rhizobia can be found as part of the normal, free-living soil microflora.

The plant partners in these symbioses belong to the family *Leguminoseae* comprised of three subfamilies: *Caesalpinoideae*, *Mimosoideae*, and *Papilionoideae* (Allen and Allen, 1981). The only non-legume known to be nodulated belongs to the genus *Parasponia* of the *Ulmaceae* family. Over 15,000 species belong to the *Leguminoseae*, ranging from tropical trees to arctic annuals, most of which can form nodules under the proper conditions (Dénarié *et al.*, 1992).

Symbiotic N_2-fixation

The formation of effective N_2-fixing root nodules is a complex and highly regulated process that requires the production and exchange of specific molecular signals between the host plant and the bacterial symbiont (Broughton *et al.*, 2000; Perret *et al.*, 2000; Spaink, 2000). This stringency entails that individual bacterial species nodulate only a particular set of host plants. The degree of host specificity

varies tremendously amongst rhizobia (Young and Johnston, 1989). Some strains like *Rhizobium leguminosarum* bv. *trifolii* have a very narrow host range, while others such as *Rhizobium* sp. strain NGR234 can nodulate over 110 different legume species, as well the nonlegume *Parasponia* (Freiberg *et al.*, 1997). *B. japonicum* strains can nodulate cowpeas, mungbeans, siratro as well as soybeans, while *S. meliloti* is known to nodulate alfalfa, sweet clover, and fenugreek.

Flavonoids: the plant signal

Phenolic compounds are known to play a role in fruit and flower coloration, insect attraction for pollination, the defense of plants from pathogens, and symbiotic N_2-fixation (Göttfert, 1993). Symbiotic N_2-fixation is initiated following the secretion of specific phenolic (flavonoid and isoflavonoid) compounds into the rhizosphere by the host plant. The (iso)flavonoid inducers excreted by the plant represents the first level of host specificity and are responsible for the induction of the bacterial genes involved in nodulation (*nod* genes). The *nod* genes of each rhizobia species are only induced by the specific (iso)flavonoid produced by its appropriate host plant (Mergaert *et al.*, 1997). In addition, certain (iso)flavonoids have the ability to repress *nod* gene expression in non-suitable symbionts. In soybeans for instance, daidzein, and to a greater extent genistein, are responsible for induction of the *B. japonicum nod* genes (Kosslak *et al.*, 1987), but they are also strong inhibitors of gene expression in *R. leguminosarum* bv. *viciae* (Firmin *et al.*, 1986) and *S. meliloti* (Györgypal *et al.*, 1991). These plant signal molecules are active at very low concentrations (10^{-7}–10^{-9} M) and have been shown to induce *nod*

genes within minutes (Djordjevic *et al.*, 1987). Some nonflavonoids such as stachydrine and trigonelline have also been shown to induce *nod* genes in *S. meliloti*. However the concentration required for induction is much greater, in the millimolar range (Phillips *et al.*, 1992; Phillips *et al.*, 1994).

Nodulation genes and their regulation

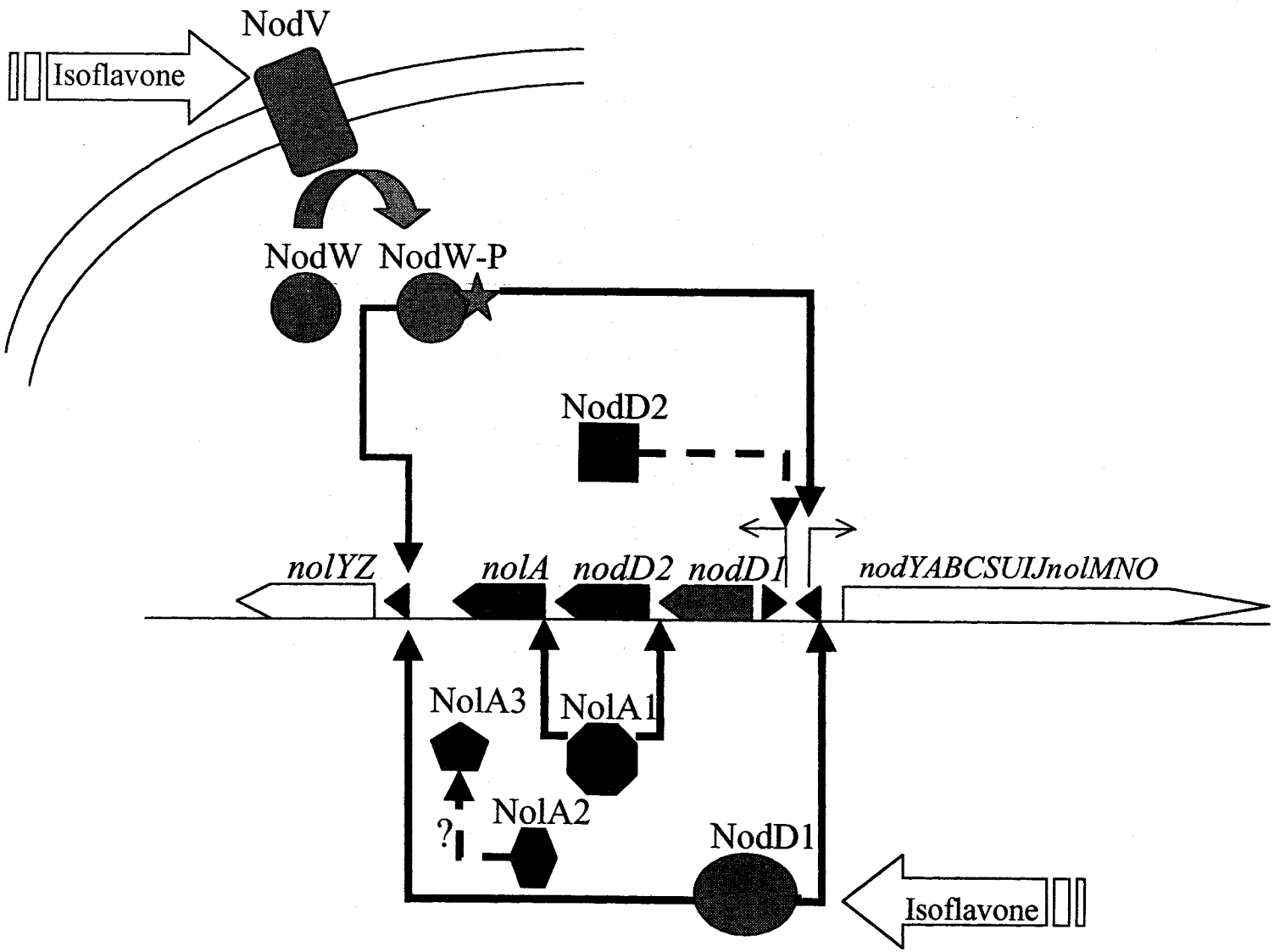
The bacterial genes involved in nodulation can be divided into two groups. The first group consists of the nodulation (*nod, nol, noe*) genes which are involved in the formation and excretion of Nod factor, the bacterial signal molecule. More than 55 *nod* genes which can be subdivided into 3 classes: common, regulatory, and host-specific (Table 1.1) have been isolated thus far and are usually located on large plasmids (pSym) alongside the genes responsible for N_2-fixation (*nif, fix*) (Galibert *et al.*, 2000). Only in *B. japonicum* and *Mezorhizobium* are these genes located on a symbiotic island of the chromosome (Kaneko *et al.*, 2002). The second group of genes include those that are involved in the production of rhizobial cell surface determinants, such as genes involved in the synthesis of exopolysaccharides (*exo* genes), lipopolysaccharides (*lps* genes), β-1,2-glucans (*ndv* genes), which are believed to play a role in the infection process (Leigh and Coplin, 1992; Breedveld and Miller, 1994; Spaink, 2000).

Regulation of the *nod* genes represents the second level of host specificity. A schematic representation of the regulation of *nod* genes in *B. japonicum* is depicted in Figure 1.1. In most rhizobia, regulation of *nod* genes by flavones is mediated by

Table 1.1. Genes involved in nodulation of *Bradyrhizobium japonicum*.

nod gene	*nod* class	Gene product function
nodA	Common	N-acetyltransferase
nodB	Common	de-N-acetylase
nodC	Common	UDP-GlcNAc transferase
nodS		S-adenosyl methionine methyl transferase
nodU		6-O-acetyl transferase
nodI	Common	ATP-binding protein
nodJ	Common	Membrane protein
nolMNO		Sugar epimerase
nodZ	Host-specific	Involved in fucosylation
nolYZ		Unknown
nolA	Regulatory	3 functionally distinct proteins $NolA_1$, A_2, A_3
$nodD_1$	Regulatory	LysR-type regulator
$nodD_2$	Regulatory	LysR-type regulator
nodVW	Regulatory	Two component regulatory system

Figure 1.1. Schematic representation of *nod* gene regulation in *Bradyrhizobium japonicum*. *nod* genes involved in nodulation are represented by block arrows, with regulatory genes shown in solid block arrows, and structural genes in open block arrows. The *nod* boxes are shown as solid triangles. Positive regulation is represented by arrows and negative regulation is represented by broken-line arrows.


NodV
Isoflavone
NodW
NodW-P
NodD2
nolYZ
nolA
nodD2
nodD1
nodYABCSUIJnolMNO
NolA3
NolA1
NolA2
?
NodD1
Isoflavone

the membrane-associated protein NodD. NodD contains a helix-turn-helix DNA binding motif consistent with transciptional regulator proteins of the LysR family. NodD is constitutively expressed (Schlaman *et al.*, 1998) at low levels and remains in an inactive form until interaction with the appropriate flavone, which activates the protein resulting in the formation of a dimer or tetramer (Wang and Stacey, 1991) which binds to a well conserved promoter sequence (*nod* box) upstream of the *nod* operons, stimulating the transcription of the remaining *nod* genes (Rostas *et al.*, 1986).

Some characteristics of the host range or host specificity can be associated with the flavonoid specificity spectrum of NodD. Rhizobia exhibiting narrow-host-range, such as *S. meliloti* and *R. leguminosarum* bv. *trifolii*, synthesize NodD proteins that respond to few flavonoids. Whereas NodD isolated from *Rhizobium* NGR234, a broad-host-range rhizobia, interacts with a much greater number of signals. Along with the sensitivity of NodD, rhizobia also differ in the number of copies of the *nodD* gene. The number of copies range from one, in *R. leguminosarum* bv. *trifolii,* to multiple copies in *B. japonicum* (2 copies) and *S. meliloti* (3 copies). While NodD remains the central regulator in *nod* gene regulation, additional regulators are known to influence expression of the *nod* genes. For instance, in *S. meliloti* a second LysR type regulator, SyrM, regulates *nod* gene expression in a flavonoid-independent fashion (Hanin *et al.*, 1998).

In *B. japonicum*, translational gene fusion reporter plasmids were used with NodD$^-$ mutant strains to demonstrate that $NodD_1$ but not $NodD_2$ was responsible for the induction of the *nod* operon (Göttfert *et al.*, 1992). However, studies performed

with *nodD*$_1$ *nodD*$_2$ double mutants showed no decreased *nod* expression which indicated an alternative pathway. Indeed, *B. japonicum nod* genes were found to also be regulated by an alternative mechanism involving a two-component regulatory system, NodVW. The transmembrane sensing protein NodV autophosphorylates upon interaction with genistein, and subsequently transfers the phosphate group to the regulatory protein NodW. Phosphorylated NodW can bind to *nod* boxes inducing expression of the *nod* genes (Loh *et al.*, 1997).

As stated previously, nodulation is a very complex and intricate balance of signals. It has been demonstrated that the strong, constitutive expression of the nodulation genes results in defective and reduced nodulation phenotypes on host plants (Knight *et al.*, 1986). Repression of the *nod* genes *in planta* has been demonstrated (Sharma and Signer, 1990; Schlaman *et al.*, 1991) but the exact mechanism of this repression remains unexplained. In *S. meliloti nod* repression is mediated by NolR, while in *Rhizobium* sp. strain NGR234 and *Bradyrhizobium elkani* NodD$_2$ acts as a repressor. Mutations to these repressors result in aberrant and delayed nodulation phenotypes (Cren *et al.*, 1995; Schlaman *et al.*, 1998). In *B. japonicum*, NolA and NodD2 form two key components in the negative regulation of the nodulation genes.

It has been demonstrated that NolA induces expression of NodD$_2$, which then acts to repress the *nod* operon (Garcia *et al.*, 1996; Fellay *et al.*, 1998). Interestingly, *nolA* encodes for three distinct proteins, NolA$_1$, NolA$_2$, and NolA$_3$ that are expressed from three in-frame ATG start codons (Loh *et al.*, 1999). The largest protein, NolA$_1$, possesses the helix-turn-helix DNA binding domain characteristic of

MerR regulatory proteins. The *nolA* gene is transcribed from two different promoters: P1 and P2. Transcription from the P1 promoter leads to the expression of $NolA_1$. The P2 promoter contains a putative $NolA_1$ binding site and drives expression of $NolA_2$ and $NolA_3$. Interestingly, a second putative $NolA_1$ binding site is found upstream of *nodD*$_2$ (Loh *et al.*, 2001) supporting the notion of $NolA_1$ regulating $NodD_2$ expression. The expression of $NolA_2$ and $NolA_3$ is dependent on the presence of $NolA_1$. The exact functions of both $NolA_2$ and $NolA_3$ are still not known, but a reduction in nodulation efficacy on soybean was observed with strong expression of $NolA_2$, while $NolA_3$ was demonstrated to be essential for nodulation of the same host (Loh *et al.*, 1999; Loh *et al.*, 2001) leading some to postulate that $NolA_2$ may function to modulate expression of *nolA*$_3$. By interacting with the stem-loop structure surrounding the *nolA*$_2$ gene, $NolA_2$ could conceivably reduce $NolA_3$ levels, thereby affecting nodulation.

It has been recently suggested that *nod* genes could also be under some form of cell density dependent or quorum sensing regulation. This would explain the previous observations that optimal expression of *nod* genes occurs at very low cell-densities (Yuen and Stacey, 1996) and increasing cell population densities results in a significant decrease in *nod* expression (Loh *et al.*, 2001). Moreover, NolA and $NodD_2$ were found to accumulate as cell population densities increased. Mutation of *nolA* resulted in derepression of the *nodYABC* operon, even at high culture densities. An extracellular signal molecule was found to be responsible for the cell density dependence. The signal molecule was isolated and chemical structure elucidated as 2-(4-{[4-(3-aminooxetan-2-yl) phenyl](imino) methyl} phenyl)

oxetan-3-ylamine, otherwise designated bradyoxetin (Loh *et al.*, 2002a). This signal was shown to accumulate at high cell densities and induce expression of $NolA_1$ through the action of NwsB, which is part of a two component regulatory system (NwsA/NwsB). NwsB is likely a homolog of NodW with which it shares 65% amino acid identity over their entire lengths. NswB also possesses the conserved helix-turn-helix DNA-binding motif typical of two-component regulator. NwsB mutants grown to high cell population densities showed low levels of *nolA* and *nodD2* expression. The expression of *nodY-lacZ* fusions in these same mutants were unaffected by changes in population density (Loh *et al.*, 2002b). This quorum sensing regulation has been proposed to be responsible for the repression of *nod* genes in nodules. The high cell-to-volume ratio in symbiosomes would replicate an environment of high population density. Using a *nodY-GUS* fusion, Loh *et al.*(2001) were able to demonstrate that *nod* gene expression remained high in nodules infected with a NolA mutant as compared to wild-type strain where the *nod* genes were repressed.

Nod factor: the bacterial signal

The end result of *nod* gene expression is the synthesis and secretion of Nod factor, the bacterial symbiotic signal to the plant. Nod factors are a key element in defining the host-range specificity. These molecules differ slightly depending on the strain of rhizobia, however the basic lipo-chito-oligosaccharide (LCO) structure remains the same (Long, 1996). Specificity is determined by minor substitutions in the degree of chitooligosaccharide polymerization, the nature of the fatty acid,

and/or oligosaccharide modifications (Mergaert *et al.*, 1997). The products of the common *nod* genes (*nodABC*) are responsible for the formation of the β-1,4-*N*-acetylglucosamine backbone. The alteration and accessorization of the basic structure is dictated by the host-specific nodulation genes, such as *nodZ*. For example, a *B. japonicum nodZ* mutant is unable to nodulate siratro, but still retains the capacity to nodulate soybeans (Stacey *et al.*, 1994).

The presence of the host-specific Nod factor in picomolar concentrations will trigger the initial stages of symbiosis in the plant, that include root hair curling and cortical cell division (Cullimore *et al.*, 2001). It has been speculated that a high affinity receptor(s) at the tip of root hairs is involved in Nod factor recognition and detection (Dénarié and Cullimore, 1993; Heidstra and Bisseling, 1996). The bacteria are then ready to begin infection of the plant roots through the formation of an infection thread, which will eventually lead to the formation of N_2-fixing nodules.

Root nodules formed by the legumes can be characterized as either determinate or indeterminate. Determinate (spherical) nodules, as found in soybeans, mung bean and common bean have a transient meristem. Indeterminate (elongated) nodules, as found on alfalfa, pea, and vetch have a persistent meristem (Pawlowski and Bisseling, 1996). As the nodule develops, the rhizobia find their way into the plant cell where they are enveloped by the plant cytoplasmic membrane (peribacteroid membrane) and differentiate into a N_2-fixing form referred to as bacteroids.

Effects of low root zone temperatures (RZT) on nodulation

Soybeans are subtropical leguminous plants requiring root zone temperatures (RZT) between 25°C-30°C for optimal symbiotic activity. Eastern Canada is at the northernmost North American limit for the production of this particular crop. In these temperate areas, low spring soil temperatures (10°C-15°C at 10 cm depth), compared with those of their native habitat, are the major limiting factor for growth and development of soybeans.

Suboptimal temperatures were found to inhibit all stages of symbiosis, with the early stages of nodule formation being especially sensitive to low RZTs (Zhang and Smith, 1994). This delay was particularly noticeable when comparing the time-course of the different steps involved in the infection process at optimal RZT (Turgeon and Bauer, 1982) and suboptimal RZT (Zhang and Smith, 1995). A short pre-incubation period (24 hours) at 25°C before transferring plants to suboptimal temperatures was shown to decrease the time required for the onset of N_2-fixation. Flavonoid levels were found to play an important role in the degree and efficiency of nodulation and N_2-fixation. The supernodulating ability of certain cultivars has been directly correlated to the increased level of flavonoids (Cho and Harper, 1991).

These findings led to the hypothesis that the inhibition of nodulation at suboptimal RZT was associated with disruption of the plant-signal molecule. In accordance with this hypothesis, the content and concentration of genistein in soybeans was found to decrease under suboptimal temperatures (Zhang and Smith, 1996a). Furthermore, using a *nodY::lacZ* gene fusion reporter plasmid, it was established that the expression of the *nod* genes required higher concentrations of

the isoflavone (genistein) at the suboptimal temperatures (Zhang *et al.*, 1996a). The addition of genistein has been proven as an effective means of overcoming the adverse effects of low RZTs on nodulation and N_2-fixation with promoted gain of yield (Zhang and Smith, 1996b). Genistein remains an expensive chemical, therefore it would be beneficial to try to find a different approach to solve the problem of delayed nodulation.

Nitrogen fixation

Once differentiated, bacteroids express the (*fix, nif*) genes required for N_2-fixation which is catalyzed by a two-enzyme complex called nitrogenase, encoded by the *nifHDK* genes (Roberts and Brill, 1981). The nitrogenase complex is composed of dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). Dinitrogenase, the larger of the two proteins is responsible for the actual reduction of N_2. Dinitrogenase reductase acts as an electron carrier and transfers electrons to the MoFe protein. Because dinitrogenase is very sensitive to oxygen, N_2-fixation must be carried out under low oxygen concentrations.

One of the strategies employed by the bacteroids to maintain a microaerobic environment suitable for N_2-fixation is the production of an oxygen binding heme protein know as leghemoglobin (Appleby, 1984). The myoglobin-like protein transports oxygen from the outer to the inner of the regions making it available for respiration, while maintaining a low level of free oxygen. The bacteroids also posses high-affinity oxidases permitting them to respire in such low oxygen environment (Delgado *et al.*, 1998). In addition nitrogenase expression itself is

indirectly regulated by means of oxygen responsive regulators such as the two-component regulatory system *fixLJ* and oxygen sensor *fixK* (Dunn, 1998).

At least 16 molecules of ATP and 8 reducing equivalents are required per molecule of N_2 reduced. Nitrogen fixation in rhizobia follows the general equation:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16\ P_i$$

Carbon metabolism

To support the high energy demand of N_2-fixation, bacteroids must be constantly supplied with a source of reduced carbon that can be oxidized to yield energy. Sucrose produced by photosynthesis is the principle source of reduced carbon that is transferred from plant shoot to root nodule cells (O'Gara *et al.*, 1989). The sucrose is then converted via the action of sucrose synthase and glycolytic enzymes to C_4-dicarboxylic acids such as malate and succinate which are provided to the bacteroids (Streeter, 1995; Vance and Heichel, 1991).

Since the early 1960s research has been carried out to elucidate the energy source used by bacteroids. Tuzimura and Meguro (1960) demonstrated early on that *B. japonicum* prefers succinate and other TCA cycle intermediates over sucrose and hexoses. In addition, subsequent experiments conducted using bacteroids isolated from soybeans demonstrated that N_2-fixation was stimulated by C_4-dicarboxylic acids but not sucrose (Stovall and Cole, 1978). Convincing evidence was supplied for this theory when multiple research groups demonstrated that mutations that

interfered with dicarboxylic acid transport essentially blocked N_2-fixation (Finan *et al.*, 1983; Ronson *et al.*, 1984; Boltan *et al.*, 1986; Watson *et al.*, 1988).

The C_4-dicarboxylic transport system (Dct) consists of a transmembrane transport protein DctA that is regulated by a two-component regulatory system, DctB (sensor kinase) and DctD (response regulator). Mutations in any one of the three components of the Dct system results in the inability to transport C_4-dicarboxylic acids and grow on C_4-dicarboxylates. While *dctA* mutants are unable to fix nitrogen, some strains mutated in *dctB* or *dctD* retain the ability to fix N_2 albeit at a reduced rate (Finan *et al.*, 1983). Once transported into the bacteroids the C_4-dicarboxylic acids are metabolized through the TCA cycle producing much needed ATP and reducing equivalents used to drive N_2-fixation (Vance and Heichel, 1991; Dunn, 1998; Poole and Allaway, 2000; Dymov *et al.*, 2004).

Tricarboxylic acid cycle

The TCA cycle is the primary energy-generating pathway that supports nitrogenase activity. In addition to energy production, the TCA cycle is also responsible for the production of reducing power and biosynthetic precursors for pathways that yield amino acids, nucleotides, and vitamins. The TCA cycle has been shown to be present in both free-living and bacteroid cells using both enzymatic activity and respirometric studies (Stovall and Cole, 1978; Rawsthorne *et al.*, 1980; Stowers, 1985; Tajima *et al.*, 1990; Streeter, 1991; Dunn, 1998).

The first step in the TCA cycle is the formation of citrate from the condensation of acetyl-CoA and oxaloacetate, a reaction catalyzed by citrate

synthase (CIS; EC 4.1.3.7). Most rhizobia rely on the oxidative decarboxylation of pyruvate by pyruvate dehydrogenase as a source of acetyl-CoA (Dunn, 1998). Several other pathways can be utilized to generate acetyl-CoA such as the degradation of poly-β-hydroxybutyrate (PHB) (Anderson and Dawes, 1990).

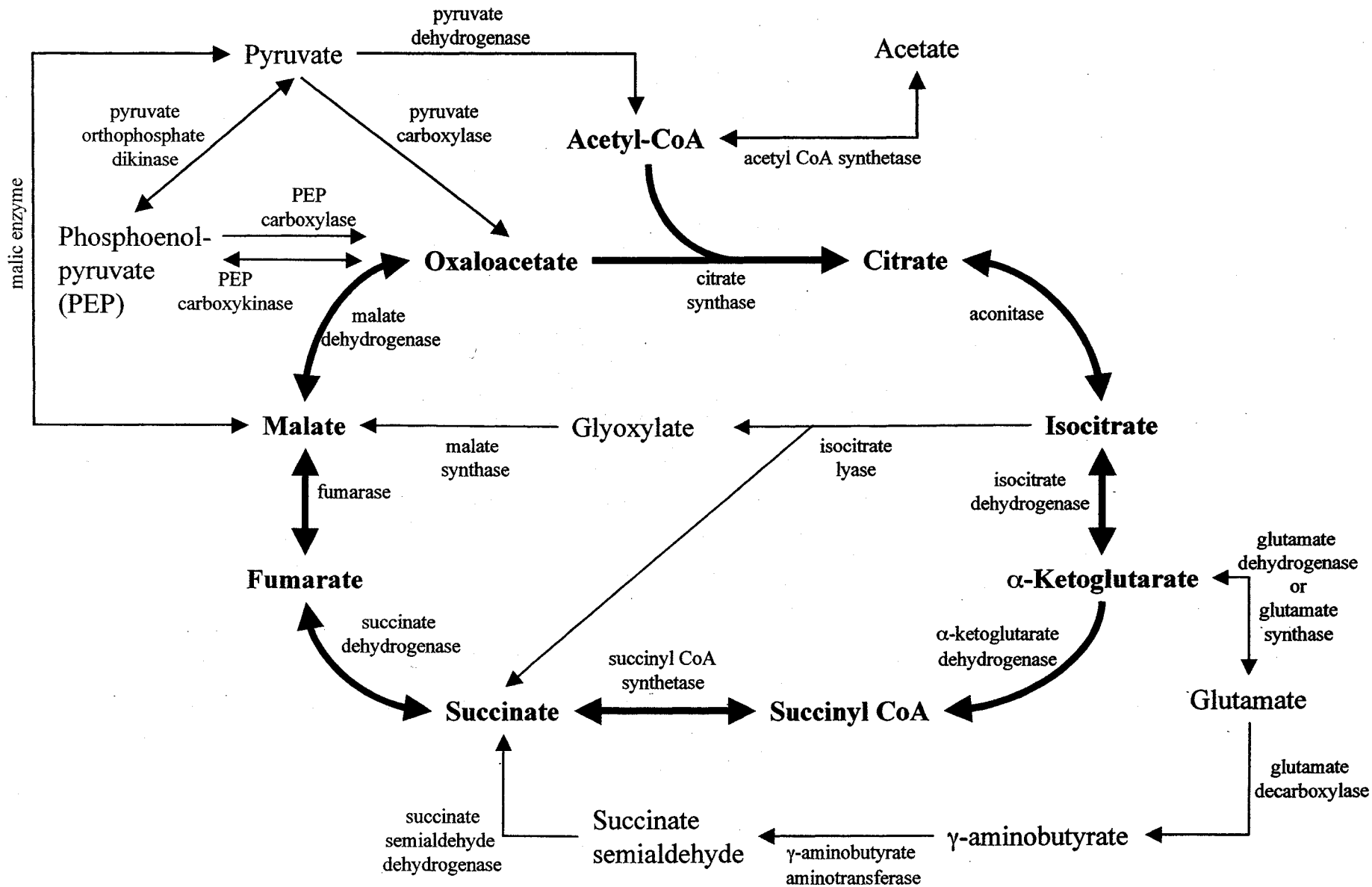
The TCA cycle then continues with the sequential action of the following enzymes: aconitase (ACN; EC 4.2.1.3), isocitrate dehydrogense (ICD; EC 1.1.1.42), α-ketoglutarate dehydrogenase also known as 2-oxoglutarate dehydrogenase (OGD; EC 1.2.4.2), succinyl-CoA synthetase also known as succinate thiokinase (SCS; EC 6.2.1.6), succinate dehydrogenase (SDH; EC 1.3.99.1), fumarase (FUM; EC 4.2.1.2), and malate dehydrogenase (MDH; EC 1.1.1.37) (Figure 1.2). The overall cycle involves the generation of oxaloacetate and CO_2 from citrate and produces per revolution: one molecule of ATP, three molecules of NADH, and one molecule of $FADH_2$, and can be summarized as follows:

$$\text{Acetyl-CoA} + 3\ NAD^+ + FAD + GDP + P_i + 2\ H_2O \rightarrow 2\ CO_2 + 3\ NADH + FADH_2 + GTP + 2\ H^+ + CoA\sim SH$$

The reducing equivalents produced by the TCA cycle can be oxidized by the electron transport chain to yield ATP. Taking this into account, the TCA cycle could generate up to 12 molecules of ATP per revolution.

Studies using mutant strains deficient in TCA cycle enzymes have demonstrated the importance of this energy producing pathway in bacteroids for effective symbiotic N_2 - fixation. Mutation in *gltA*, the gene encoding citrate

Figure 1.2. The reactions involved in the tricarboxylic acid (TCA) cycle. The arrows indicate the direction of the reaction, and double-headed arrows represent reversible reactions. The enzymes responsible for the reactions are shown inside the circle. The products generated by the reactions are depicted between the arrows.


Pyruvate
pyruvate dehydrogenase
Acetate
pyruvate orthophosphate dikinase
pyruvate carboxylase
Acetyl-CoA
acetyl CoA synthetase
malic enzyme
PEP carboxylase
Phosphoenol-pyruvate (PEP)
PEP carboxykinase
Oxaloacetate
citrate synthase
Citrate
malate dehydrogenase
aconitase
Malate
Glyoxylate
Isocitrate
malate synthase
isocitrate lyase
fumarase
isocitrate dehydrogenase
glutamate dehydrogenase or glutamate synthase
Fumarate
α-Ketoglutarate
succinate dehydrogenase
α-ketoglutarate dehydrogenase
succinyl CoA synthetase
Succinate
Succinyl CoA
Glutamate
glutamate decarboxylase
succinate semialdehyde dehydrogenase
Succinate semialdehyde
γ-aminobutyrate aminotransferase
γ-aminobutyrate

synthase in *S. meliloti* resulted in glutamate auxotrophy and the formation of small bacteroid-free nodules that expressed a Fix$^-$ symbiotic phenotype (Kahn *et al.*, 1995). A *gltA* mutation in *Sinorhizobium fredii* USDA257 also resulted in glutamate auxotrophy and nodules unable to fix N_2 (Krishnan *et al.*, 2003). On the other hand, *Rhizobium tropici*, which possesses two citrate synthases, one chromosomal (*ccsA*) and a second encoded on a plasmid (*pcsA*), only produced defective nodules if both genes were knocked out (Hernández-Lucas *et al.*, 1995).

In *E. coli*, aconitase is encoded by a pair of paralogous genes *acnA* and *acnB* (Jordan *et al.*, 1999). Although, little is known about aconitase in rhizobia, an *acnA* homologue has been identified in *B. japonicum*. Mutant *acnA* strains of *B. japonicum* were found to retain substantial aconitase activity and demonstrated a wild-type symbiotic phenotype, strongly suggesting the presence of a *acnB*-like homologue (Thöny-Meyer and Künzler, 1996).

Similar to CIS mutants, mutants defective in ICD demonstrated glutamate auxotrophy and induced the formation of nodules defective in N_2-fixation (McDermott and Kahn, 1992). Interestingly, while both CIS and ICD single mutants form Fix$^-$ nodules, *S. meliloti* mutants deficient in both enzymes are unable to form nodules at all (McDermott and Kahn, 1992).

Mutants defective in OGD isolated from both *R. leguminosarum* bv. *viciae* and *S. meliloti* were shown to generate nodules unable to fix N_2 on pea and alfalfa plants respectively (Johnson *et al.*, 1966; Duncan and Fraenkel, 1979). The glyoxylate shunt was suggested to be an alternative pathway employed by *sucA* mutant of *B. japonicum* defective in OGD that retained the ability, although

delayed, to form effective symbiotic nodules and were also able to use succinate and malate as sole carbon source (Green and Emerich, 1997a, b). The delay in nodulation events observed with this mutants suggest that OGD has a greater impact on early stages of nodulation and is not necessary for symbiotic N_2-fixation. Enzyme assays conducted on isocitrate lyase, an enzyme involved in the glyoxylate shunt, have revealed low levels of activity in *B. japonicum* and *S. meliloti* (Duncan and Fraenkel, 1979; Green *et al.*, 1998). In addition, isocitrate lyase activity levels were not detectable in *S. meliloti* or *R. leguminosarum* bacteroids, and only low activity was detected in *B. japonicum* bacteroids. These observations would tend to indicate that the glyoxylate shunt plays a limited role at best in bacteroids (Johnson *et al.*, 1966)

A *R. leguminosarum* SCS mutant (*sucD*) isolated using Tn*5* mutagenesis was found to have a $Nod^+ Fix^-$ symbiotic phenotype on pea plants. (Walshaw *et al.*, 1997). A reduction in SCS activity was observed but the levels remained high enough to suggest the presence of a paralog. Because the genes encoding SCS (*sucCD*) are located directly upstream of the genes encoding OGD (*sucAB*) it remains unclear if the phenotype observed was the result of polar effects on OGD. Therefore until mutants that are only defective SCS are isolated the exact role that SCS plays in N_2-fixation will remain uncertain.

E. coli encodes three distinct fumarase that can be further categorized into two classes: Class I (FumA, FumB) fumarases are oxygen-labile, heat-stable enzymes involved in the TCA cycle (Cronan and Laporte, 1996), whereas Class II (FumC) fumarases are oxygen-stable, heat-labile enzymes involved in oxidative

stress protection (Liochev and Fridovich, 1992). A fumarase gene cloned from *B. japonicum* showed significant homology to Class II and therefore was designated *fumC*. Deletional mutation of *fumC* in *B. japonicum* did not completely inhibit fumarase enzyme activity nor did it impair the ability to form effective nodules. Further investigation revealed that the residual fumarase activity was heat-stable, suggesting the presence of additional class I fumarase paralog(s) either FumA and/or FumB (Acuña *et al.*, 1991).

The last step in the TCA cycle involves the oxidation of malate to oxaloacetate that can then be reintroduced into another cycle of the energy generating pathway. This final reaction of the TCA cycle is catalyzed by MDH encoded by the *mdh* gene. In *S. meliloti* bacteroids, MDH activity levels have been shown to be elevated (Driscoll and Finan, 1993) and even upregulated during symbiosis (Natera *et al.*, 2000). Previously thought to be lethal in *S. meliloti* due to polar effects on SCS and OGD, the use of Tn*5*tac1 mutagenesis has recently permitted Dymov *et al.* (2004) to isolate an *mdh* mutant. The mutant demonstrated reduced growth on all carbon sources, with arabinose and glutamate showing the greatest reduction of growth rates. The Mdh^- mutant proved to be able to nodulate alfalfa but the nodules were unable to fix N_2 (Dymov *et al.*, 2004).

Succinate dehydrogenase

Succinate dehydrogenase, also known as succinate:ubiquinone oxidoreductase or complex II due to its additional role in aerobic respiratory chain, is an important enzyme involved in the TCA cycle. As the only membrane bound

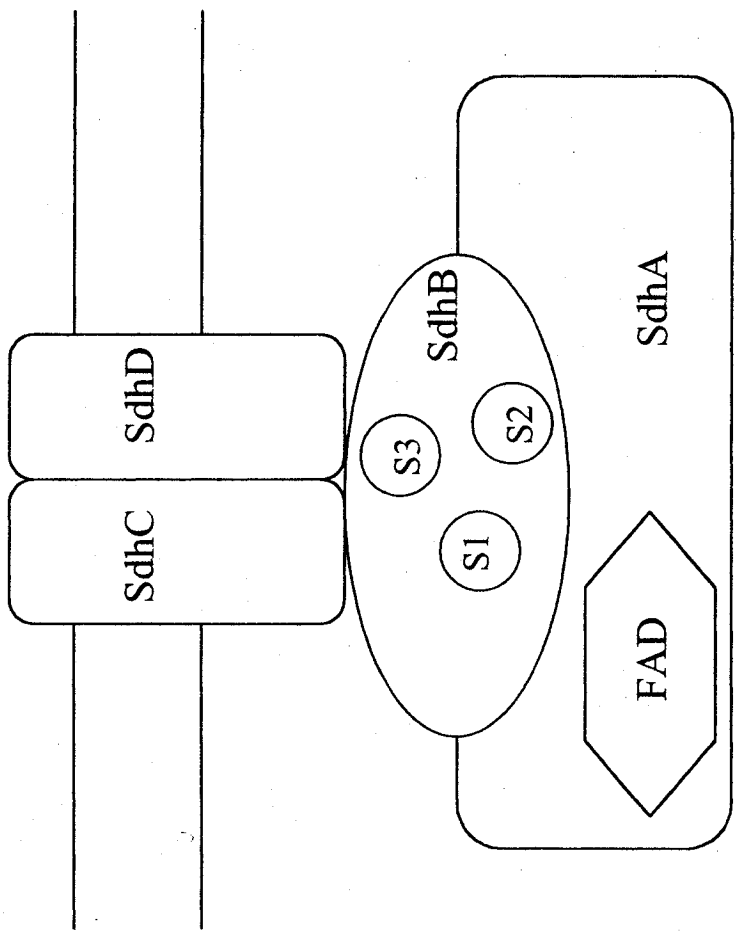
enzyme of the TCA cycle, SDH catalyzes the coupled reaction involving oxidation of succinate to fumarate and subsequent reduction of ubiquinone to ubiquinol. SDH is closely related to fumarate reductase (FRD) which catalyses the reduction of fumarate to succinate under anaerobic respiration in *E. coli* (Guest, 1981).

SDH has been purified from numerous species from all three domains, and appears to be highly conserved (Onishi, 1987). Little is known about SDH in rhizobia. Most of our knowledge of SDH comes from studies on *E. coli*, *B. subtilis* and beef heart mitochondrial SDHs (Ackrell *et al.*, 1992; Kita *et al.*, 1989).

The genes encoding for the SDH subunits have been cloned and sequenced from various bacteria: *E. coli* (Darlison and Guest, 1984; Wood *et al.*, 1984), *B. subtilis* (Magnusson *et al.*, 1986; Phillips *et al.*, 1987) and *B. japonicum* (Westenberg and Guerinot, 1999). In most eubacterial systems including *B. japonicum*, the SDH enzyme complex is composed of four subunits (Figure 1.3): two large hydrophilic catabolic subunits (SdhA, SdhB) and two smaller hydrophobic subunits (SdhC, SdhD) acting as membrane anchor proteins (Westenberg and Guerinot, 1999).

The SDH subunits are encoded by the *sdhCDAB* operon (Bachmann, 1990; Westenberg and Guerinot, 1999). The gene order is slightly different in the archeal *sdhABCD* operon (Schäfer *et al.*, 2002). In *B. subtilis*, the SDH complex is only composed of three subunits (SdhA, SdhB, SdhC) encoded by the *sdhCAB* operon. In this case, it is believed that the two membrane anchors found in bacteria (SdhC and SdhD) fused to become a single membrane anchor subunit (Hederstedt, 1999).

Figure 1.3. Schematic representation of the succinate dehydrogenase enzyme complex. FAD is shown in association with the flavoprotein SdhA. The three iron-sulfer clusters of the iron sulfur protein SdhB are represented as S1, S2, and S3. The hydrophobic subunits SdhC and SdhD are depicted as transmembrane proteins in association with each other, as well as the two catabolytic subunits through SdhB.


SdhD
SdhC
SdhB
S3
S2
S1
SdhA
FAD

In all cases, SdhA consists of a flavoprotein subunit containing a covalently-bound flavin adenine dinucleotide (FAD) moiety. The FAD was found to be attached to SdhA through an 8α-*N*3-histidyl-FAD linkage (Walker and Singer, 1970). The formation of this linkage is a complex process believed to be self-catalyzed and requires the proper folding of the flavoprotein in order to occur (Mewies *et al.*, 1998; Efimov *et al.*, 2001). SdhA is the site of dicarboxylate binding and is believed to be the catalytic site of succinate oxidation and reduction of FAD to $FADH_2$. The electrons from $FADH_2$ are sequentially transferred to iron-sulfur clusters of the second catabolic subunit, SdhB.

SdhB is an iron-sulfur protein containing three iron-sulfur clusters (S1[2Fe-2S],S2[4Fe-4S],S3[3Fe-4S]). S2 was initially believed to not be part of the electron transfer system but rather to play a regulatory or structural role in the enzyme complex (Ackrell *et al.*, 1992; Singer and Johnson, 1985), however, X-ray structure analysis revealed that it was actually part of a linear electron transport chain between FAD and ubiquinone. Furthermore, the close physical association of the clusters (~ 11 Å) suggest that they all participate in the electron transfer (Ohnishi *et al.*, 2000). The catalytic subunits are attached to the inner side of the cytoplasmic membrane by one or two membrane anchor proteins, and unlike the highly conserved catabolic subunits the membrane anchor subunits show a greater degree of amino acid sequence variation among organisms.

In *E. coli* the two hydrophobic heme b-containing subunits that anchor the enzyme complex are encoded by *sdhC* and *sdhD*. SdhC and SdhD are also believed to be components of cytochrome b_{556} (Murakami *et al.*, 1985). Each anchor protein

contains three membrane-spanning helices. In cases when only one anchor protein is present, such as found in *B. subtilis*, the protein consists of five membrane-spanning helices. Experiments conducted with *E.coli sdhC* and *sdhD* mutant strains demonstrated that both anchors are needed for quinone:oxidoreductase activity involved in the electron transport chain. However the two hydrophilic subunits (SdhA and SdhB) alone are sufficient for SDH activity when assayed with artificial electron acceptors and donors (Nakamura *et al.*, 1996).

A *R. leguminosarum* mutant, GFS18, defective in SDH activity was isolated using *N*-methyl-N'-nitro-*N*-nitrosoguanidine (NTG) mutagenesis after attempts to generate mutants through Tn*5* transposon mutagenesis failed. GFS18 had reduced SDH activity and was able to grow on glucose and fumarate but not succinate as sole carbon source. Succinate uptake assays revealed that the phenotype was linked to SDH activity and not a succinate transport deficiency. When inoculated on alfalfa plants, GFS18 was able to nodulate, however the bacteria were unable to differentiate into bacteroids resulting in a Fix^- symbiotic phenotype (Finan *et al.*, 1981). Similarly, a *S. meliloti sdh* mutant, isolated using NTG mutagenesis was unable to use succinate, glutamate, acetate, pyruvate or arabinose as a sole carbon source, while retaining the ability to grow on minimal media with glucose or fumarate. No SDH activity was detected in the mutant which was able to nodulate alfalfa plants but not fix N_2 (Gardiol *et al.*, 1982). The Fix^- phenotype was further supported by microscopic observations demonstrating the absence of bacteroids in nodules (Gardiol *et al.*, 1987).

Regulation of the *sdh* operon

The majority of our knowledge of *sdh* gene regulation comes from studies on the *E. coli* and mitochondrial systems. Very little is known about how these genes are regulated in rhizobia, with *B. japonicum* being the best characterized system thus far. In *E. coli*, the *sdh* operon is expressed from a single promoter located 219 bp upstream of the first gene in the operon *sdhC* (Park *et al.*, 1995). Expression of the operon is optimal under aerobic conditions, with a ten-fold reduction in expression occurring under anaerobic conditions (Park *et al.*, 1995).

Studies using *lacZ* fusions have attributed the repression of the *sdh* operon to the combined affects of the ArcA/ArcB two component regulatory system and FNR (Iuchi *et al.*, 1994; Park *et al.*, 1995). Under anaerobic or microaerobic conditions, ArcB undergoes autophosphorylation and then catalyzes the transphosphorylation of ArcA. Phosphorylated ArcA then binds to the *sdh* operon at four sites near the promoter region preventing expression (Shen and Gunsalus, 1997).

The *B. japonicum sdh* operon appears to be regulated by a different mechanism, as *sdh* expression increases under reduced oxygen conditions. This is not at all surprising considering that the TCA cycle must operate under microaerophillic conditions to support symbiotic N_2-fixation. The transcriptional start site of the *B. japonicum sdh* operon has been determined to be 56 bp upstream from the *sdhC* initiation codon (Westenberg and Guerinot, 1999). The mechanisms of *sdh* regulation in rhizobia remains to be elucidated.

This study

The establishment of symbiosis between legumes and rhizobia has been shown to be elongated in the suboptimal temperatures of temperate soils, especially early in the growing season (Zhang and Smith, 1994). Although all stages involved in the establishment of nitrogen fixation have been reported to be inhibited by low RZT (Matthews and Hayes, 1982; Lynch and Smith, 1994), the most sensitive steps appears to be the production and detection of the plant signal molecule (Zhang and Smith, 1994). The application of genistein has been proven an effective means of overcoming the adverse effects of low RZT on nodulation and N_2-fixation (Zhang and Smith, 1996b). This work was undertaken to investigate *nod* gene regulation in *B. japonicum* under suboptimal RZT. Our hypothesis was that mutants in which the *nod* genes are expressed in the absence of the plant *nod* gene induction signals might similarly be able to overcome the low RZT inhibition of nodulation, and would then be a good alternative to the direct application of genistein in the field. Mutants with altered *nod* gene regulation were isolated and characterized.

Our interest in regulation of symbiotic N_2-fixation was extended to carbon metabolism of rhizobia via the TCA cycle. N_2-fixation remains an extremely energy-expensive reaction and requires the bacteria to produce large amounts of energy and reducing power under oxygen-limited conditions to sustain the reaction. It has been shown that rhizobia most likely meet these requirements through the oxidation of C_4-dicarboxylic acids, such as malate and succinate via the TCA cycle (Vance and Heichel, 1991). Because of their critical role in symbiosis, the TCA cycle enzymes have received a great deal of attention over the years, however, our

understanding of the overall genetic regulation of the different enzymes is limited. SDH activity has been shown to decrease under suboptimal temperature. Cold-sensitive *Rhizobium* mutants demonstrated decreased SDH activity under suboptimal temperatures compared to cold acclimated rhizobial strains (Sardesai and Babu, 2000). This project was involved in characterizing the *sdh* operon and generating *sdh*$^-$ mutants with the general objective of increasing our understanding of basic gene regulation in this system.

During the course of this investigation we were also able to isolate endophytic bacteria from soybean roots that demonstrated potential as plant growth-promoting bacteria (PGPB). Identification and characterization of these strains were carried out and presented in an appendix of this thesis.

Chapter 2. Materials and methods

Bacterial strains and growth media

All bacterial strains, plasmids and transposons used in this study are listed in Table 2.1. Frozen permanents of the cultures were prepared by combining late log phase liquid culture with an equal volume of medium containing 14% dimethylsulfoxide (DMSO) and stored at -80°C. Bacterial strains were incubated at 30°C (*B. japonicum* and *S. meliloti*) or 37°C (*E. coli*) (unless otherwise indicated). Broth cultures were grown in Erlenmeyer flasks with shaking (200 rpm) or in 16 × 150-mm test tubes on a Rollerdrum (New Brunswick Scientific Co., Edison, NJ).

Growth media were sterilized by autoclaving at 121°C for 20 min. *B. japonicum* strains were cultured in either yeast mannitol (YM), yeast salts (YS), or tryptone yeast extract (TY). YM medium contained per litre: K_2HPO_4, 0.5 g; $MgSO_4$, 0.1 g; NaCl, 0.1 g; mannitol, 10 g; yeast extract, 1.0 g; and $CaCl_2 \cdot H_2O$, 0.1 g (Somasegaran and Hoben 1994). YS contained per litre: K_2HPO_4, 0.23 g; Na-glutamate, 0.1 g; yeast extract, 1.0 g; glycerol, 4.0 g and supplemented after sterilization with: 1 M $MgSO_4$, 407 µl; 0.5 M $CaCl_2$, 68 µl; 1000X vitamin stock, 1.0 ml; 1000X trace elements, 1.0 ml. 1000X vitamin stock was stored in the dark and contained per litre: riboflavin, 20 mg; p-aminobenzoic acid, 20 mg; nicotinic acid; 20 mg; biotin, 20 mg; thiamine-HCl, 20 mg; pyridoxine-HCl, 20 mg; Ca-pantothenate, 20 mg; and inositol, 120 mg. 1000X trace elements stock contained per litre: H_3BO_3, 145 mg; $FeSO_4$, 125 mg; $MnCl_2$, 4 mg; $ZnSO_4$, 108 mg; Na_2MoO_4, 125 mg; and nitrilotriacetate, 7.0 g (Bishop *et al.*, 1976). TY contained per litre:

Table 2.1: Bacterial strains, plasmids, and transposons used in this study*.

Strain, plasmid, or transposon		Relevant characteristics	Reference or source
B. japonicum			
	USDA110	Wild-type	G. Stacey
	ZB977	USDA110 (pZB32)	G. Stacey
	Bj30050	UV mutants derived from USDA110, putative nod gene expression	This study
	Bj30051		This study
	Bj30052		This study
	Bj30053		This study
	Bj30054		This study
	Bj30055		This study
	Bj30056		This study
	Bj30057		This study
	Bj30058		This study
	Bj30059		This study
	Bj30050yz	Bj30050 (pZB32)	This study
	Bj30051yz	Bj30051 (pZB32)	This study
	Bj30052yz	Bj30052 (pZB32)	This study
	Bj30053yz	Bj30053 (pZB32)	This study
	Bj30054yz	Bj30054 (pZB32)	This study
	Bj30055yz	Bj30055 (pZB32)	This study
	Bj30056yz	Bj30056 (pZB32)	This study
	Bj30057yz	Bj30057 (pZB32)	This study
	Bj30058yz	Bj30058 (pZB32)	This study
	Bj30059yz	Bj30059 (pZB32)	This study
S. meliloti			
	Rm1021	SU47 str-21	Meade *et al.*, 1982
	RmG212	Rm1021 Lac$^-$	J. Glazebrook
	Rm30168	Rm1021 *sdhC172*::EZ::TN	This study
	Rm30169	Rm1021 *sdhD280*::EZ::TN	This study
	Rm30170	Rm1021 *sdhC80*::EZ::TN	This study
	Rm30181	Rm30168 (pFD71)	This study
	Rm30183	Rm30169 (pFD71)	This study
	Rm30186	Rm30170 (pFD71)	This study
	Rm30187	RmG212 *sdhC44*::*lacZ* transcriptional fusion containing pFD60, Nm^r	This study

	Rm30188	RmG212 *sdhC375*:: *lacZ* transcriptional fusion containing pFD61, Nm^r	This study
E. coli			
	S17.1	Sm^rKm^s, chromosomaly intergrated *tra* and *mob* genes	Simon *et al.*, 1983
	S-17-1/λpir	RK2 *tra* regulon, pir, host for pir-dependent plasmids	Simon *et al.*, 1983
	DH5α	F^- *endA1 hsdR17* (r_k^- m_k^-) *supE44 thi-1 recA1 gyrA96 relA1* Δ(argF-lacZYA) U169 Φ80d*lacZ* ΔM15 λ^-	BRL Inc.
	DH5α/λ*pir*	λ*pir*, host for *pir*-dependent plasmids	T. Charles
	MT607	*pro-82 thi-1 endA esdR17 supE44 recA56*	Finan *et al.*, 1986
	MT609	*thyA36 polA1*, Sp^r	T. Finan
	MT614	MT607ΩTn*5*	Finan *et al.*, 1986
	MT616	MT607 (pRK600)	Finan *et al.*, 1986
	G312	MT607Ω5::Tn*5*-B20	Driscoll and Finan, 1997
Plasmids			
	pLAFR1	IncP cosmid cloning vector; Tc^r	Friedman *et al.*, 1982
	pRK7813	RK2 derivative, pUC9 polylinker; Tc^r	Jones and Gutterson, 1987
	pRK600	pRK2013 *npt*::Tn*9*; Cm^r	Finan *et al.*, 1986
	pVIK112	R6KoriV promoterless *lacZ* suicide vector, Km^r	Kallogeraski and Winans, 1997
	pZB32	*nodY::lacZ*, Tc^r	Banfalvi *et al.*, 1988
	pJQ200mp18	*sacB*; Gm^r	Quandt & Hynes, 1993
	pJQ200sk	*sacB*; Gm^r	Quandt & Hynes, 1993
	PPH1JI	IncP plasmid, Gm^r,Sp^r,Cm^r	Beringer *et al.*, 1978
	pGEM-T Easy	Amp^r	Promega
	pFD09	pGEM-T Easy with *sdhC*	This study
	pFD10	pGEM-T Easy with *sdhD*	This study
	pFD11	pGEM-T Easy with *sdhB*	This study
	pFD12	pRK7813 with *Not*I fragment of pFD09	This study
	pFD13	pRK7813 with *Not*I fragment of pFD10	This study
	pFD14	pRK7813 with *Not*I fragment of pFD11	This study

pFD15	pGEM-T Easy with -713 *sdhC* to *sdhA262 S. meliloti* fragment	This study
pFD17	pFD12::Tn*5*	This study
pFD18	pFD12::Tn*5*	This study
pFD19	pFD12::Tn*5*	This study
pFD20	pFD12::Tn*5*	This study
pFD23	pFD15::EZ::TN	This study
pFD24	pFD15::EZ::TN	This study
pFD25	pFD15::EZ::TN	This study
pFD26	pFD15::EZ::TN	This study
pFD27	pFD15::EZ::TN	This study
pFD28	pFD15::EZ::TN	This study
pFD51	pGEM-T Easy with *Eco*RI fragment of pFD23	This study
pFD52	pGEM-T Easy with *Eco*RI fragment of pFD24	This study
pFD53	pGEM-T Easy with *Eco*RI fragment of pFD25	This study
pFD54	pGEM-T Easy with *Eco*RI fragment of pFD26	This study
pFD55	pGEM-T Easy with *Eco*RI fragment of pFD27	This study
pFD56	pGEM-T Easy with *Eco*RI fragment of pFD28	This study
pFD60	pVIK112 with -530 bp upstream of *sdhC* to *sdhC44* fragment of Rm1021, Km^r	This study
pFD61	pVIK112 with -24 bp upstream of *sdhC* to *sdhC375* fragment of Rm1021, Km^r	This study
pFD62	pJQ200sk with *Not*I fragment of pFD51	This study
pFD63	pJQ200sk with *Not*I fragment of pFD52	This study
pFD64	pJQ200sk with *Not*I fragment of pFD53	This study
pFD65	pJQ200sk with *Not*I fragment of pFD54	This study
pFD66	pJQ200sk with *Not*I fragment of pFD55	This study
pFD67	pJQ200sk with *Not*I fragment of pFD56	This study
pFD71	pLARF1 clone, isolated by complementation of Rm30168	This study

	pFD123	pFD15*sdhC149*::Tn*5*-B20	This study
	pFD127	PFD15*sdhD59*::Tn*5*-B20	This study
	pFD150	pFD12pRK7813::Tn*5*	This study
	pFD151	pFD14*sdhB386*::Tn*5*	This study
	pFD152	pFD14*sdhB717*::Tn*5*	This study
	pFD154	pFD14*sdhB185*::Tn*5*	This study
Tranposons			
	EZ::TN	Km^r	Epicentre
	Tn*5*	Km^r, Sm^r	Berg and Berg, 1986
	Tn*5*tac1	Km^r, P_{tac} outward oriented	Chow and Berg, 1988
	Tn*5*-B20	Km^r, promoterless *lacZ*	Simon *et al.*, 1989

*Abbreviations: Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nm, neomycin; Tc, tetracycline; Sp, spectinomycin; Sm, streptomycin; Gm, gentamycin; Rf, rifampicin.

125 mg; and nitrilotriacetate, 7.0 g (Bishop *et al.*, 1976). TY contained per litre: Bacto tryptone, 5.0 g; yeast extract, 3.0 g; and $CaCl_2 \cdot H_2O$, 0.44 g (Beringer, 1974).

E. coli strains were grown in either LB or TY medium. LB contained per litre: Bacto tryptone, 10 g; yeast extract, 5.0 g; and NaCl, 5.0 g (Beringer, 1974). *S. meliloti* strains were grown in LBmc or TY medium. LBmc consisted of LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (Glazebrook and Walker, 1991). Solid media was prepared by the addition of 1.5% (wt/vol) Difco Bacto agar (Difco Laboratories, Detroit).

Modified M9 minimal salt medium was used as the defined medium in carbon source studies (Miller, 1972). M9 medium was prepared as a 2x stock and contained per litre: Na_2HPO_4, 2.9 g; KH_2PO_4, 1.5 g; NaCl, 0.25 g; and NH_4Cl, 0.5 g. After autoclaving M9 was supplemented with 0.25 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 0.15 mg/l of biotin. All carbon sources were filter sterilized using 0.45 μm syringe filters (Fisher Scientific) and added to M9 media at a final concentration of 15 mM.

Antibiotics solutions were filter sterilized (45 μm filters) and stored at 4°C. Unless otherwise stated in the text, the following antibiotic concentrations (μg/ml) were used with *B. japonicum* strains: chloramphenicol, 25; rifampicin, 50; and tetracycline 100 (20 for plasmid maintenance). In the case of *E. coli* and *S. meliloti* strains, the antibiotic concentration used were as follows: ampicillin, 100; chloramphenicol, 10; kanamycin, 20; gentamycin, 20; neomycin, 200; spectinomycin, 100; streptomycin, 200; and tetracycline, 10. For the auxotroph *E. coli* MT609, the growth media was supplemented with thymidine at a final concentration of 60 μg/ml. 5% sucrose was used for levansucrase induction with the

vector pJQ200mp18 (Quandt and Hynes, 1993). 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was prepared in DMSO (80 mg/ml) and used at a concentration of either 40 or 80 $\mu g \cdot ml^{-1}$. Genistein (4', 5, 7-trihydroxyisoflavone; Upstate Biotechnology, Lake Placid, NY, U.S.A.) was prepared in methanol and used at the indicated concentrations.

Bacterial Conjugation

Broth cultures of the donor, recipient and mobilizer (if needed), were grown overnight in liquid medium with the appropriate antibiotics and growth factors. The mid- to late-log-phase cultures were washed twice in sterile 0.85% NaCl, resuspended in fresh medium and mixed together in equal volumes. An aliquot (10-50 µl) of mating mix was spotted onto an LB or TY plate, allowed to dry and incubated overnight at 30°C. The resulting mating spots were resuspended in 1 ml 0.85% NaCl, and dilutions were spread onto media containing the appropriate antibiotics. For the simple transfer of a plasmid between strains, the conjugation was carried out by patch mating. Colonies from plates of donor, recipient and mobilizer were mixed together with a sterile stick directly on an LB or TY plate, and incubated overnight at 30°C. Patches were then streaked isolated onto media containing the appropriate antibiotics.

Preparation of electrocompetent *B. japonicum* cells

A fresh culture of the strain to be electroporated was grown in YM or LBmc at 30°C to an A_{600} of 0.4- 0.6. Cells were chilled on ice for 15-30 min and then

harvested by centrifugation at 10,000 × g for 10 min at 4°C. The pellet was washed four times with ice-cold sterile ddH_2O, once with cold 10% glycerol, and then resuspended in 10% glycerol to an approximate 10^{10} - 10^{11} CFU · ml^{-1}. The cells were kept on ice until used (Guerinot *et al.*, 1990).

Electroporation

Electrocompetent cells (90 µl) were mixed with 2 µg of plasmid DNA by vortexing at high speed for 10 s and then chilled on ice for 30 min. The cells were then loaded into a pre-chilled electroporation cuvette (Bio-Rad) and electroporated using a Gene Pulser transfection apparatus (Gene Pulser; Bio-Rad Laboratories, Mississauga, ON). The cells were pulsed using a 12.5kV·cm^{-1} field for a period of 5 to 8 ms. Following electroporation, the cells were immediately suspended in 1 ml of YM broth and incubated overnight.

***E. coli* competent cell preparation and transformation**

The $CaCl_2$ method was used to prepare competent cells (Sambrook, *et al.*, 1989). An overnight culture grown from a single colony was sub-cultured in LB supplemented with 15 mM glucose to an A_{600} of approximately 0.5. The cells were placed on ice for 10 min, centrifuged at 3,020 × g at 4°C for 5 min, resuspended in 10 ml of cold solution A (20 ml 0.5 M $CaCl_2$, 80 ml sterile ddH_2O), chilled on ice for 1h, then cells were then centrifuged at 3,020 × g at 4°C for 10 min. The pellet was gently resuspended in 1ml of ice cold solution B (9ml solution A, 1ml sterile glycerol), and 200 µl aliquots were stored at -80°C.

Transformation was done by gently mixing 2.5 µl pre-chilled DNA with 200 µl competent cells that had been thawed on ice, and incubating on ice for 30 min. The cells were heat shocked at 42°C for 90 s then immediately placed on ice for 60 s. 1 ml of pre-warmed LB was added, and the tubes placed on a shaker (200 rpm) for 45 min at 37°C. The cells were pelleted by centrifuging for 10 min at 4,000 × g, the supernatant was discarded, and the pellet was resuspended in 100 µl LB which was spread onto LB plates containing the appropriate antibiotic.

β-Galactosidase activity assay

The method was adapted from the permeablized cell β-galactosidase assay described by Miller (1972). Bacterial cultures were aliquoted into Z buffer to a final volume of 4 ml and an A_{675} of approximately 0.1. 1 ml of the suspended cells was added to each of the three tubes for triplicate measurements. The remaining 1 ml of suspended cells was placed in a cuvette and the A_{675} was measured versus a blank as reference. Two tubes were filled with Z buffer for use as blanks. The cells in each tube were permeabilized by the addition of 10 µl chloroform and 5 µl 0.1% SDS followed by light vortexing. The tubes were equilibrated in a 30°C water bath for at least 10 min. 4 mg/ml of ONPG (o-nitrophenyl-β-galactopyranoside) was dissolved in Z buffer 30 min prior to assaying, wrapped in aluminum foil to protect it from light. The assays were initiated by adding 200 µl of ONPG solution. The reactions were stopped when the yellow colour appeared to be between A_{420} 0.2-0.6 by adding 0.5 ml of 1 M Na_2CO_3, and the assay time was recorded. The tubes were centrifuged for 10 min at 1,100 × g and the A_{420} of the supernatant was determined.

The blank reactions were used to set the autozero to cancel out the effects of spontaneous cleavage of ONPG. Miller units were calculated as follows:

$$\text{Miller Units (MU)} = \frac{1000 \times A_{420}}{(\text{time [min.]} \times A_{675})} \qquad \text{(Miller, 1972)}$$

Z buffer was adjusted to pH 7, not autoclaved, and contained per litre: $Na_2HPO_4 \cdot 7H_2O$, 16.1 g; $NaH_2PO_4 \cdot H_2O$, 5.5 g; KCl, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.246 g; and β-mercaptoethanol, 2.7 ml. The β-mercaptoethanol was added just prior to using the Z buffer.

Transcriptional fusion assays

For carbon source expression comparison, strains to be evaluated were grown to late log phase (A_{600} 0.8- 1.0) in 5 ml M9 media supplemented with 15 mM of the tested carbon source and assayed in triplicate for β-galactosidase activity as described above. For growth kinetics studies, strains were grown in 250 ml of M9 media with 15 mM carbon source of interest. 5 ml samples were taken at intervals and stored on ice until β-galactosidase activity was determined.

Growth curve studies

Overnight cultures of the bacterial strains were grown in 5ml LBmc. The cultures were washed once with 0.85% saline and resuspended in M9 medium. These cultures were used to inoculate 3 replicate cultures containing 5 ml of M9

medium with the carbon source of interest. Growth was followed by measuring the change in A_{600} at indicated intervals using a Bausch and Lomb Spectronic 21 (Bausch and Lomb, USA).

Symbiotic plant assay assemblies

Modified Leonard assemblies were constructed as described by Leonard (1943). Before assembly, the pots and beakers to be employed were carefully washed to remove all organic matter. Soybean plants were grown in 13 cm diameter pots and alfalfa plants were grown in 9 cm diameter pots. Lateral holes on the pots were plugged with electrical tape and a cotton wick penetrated the pot from the central bottom hole. The pots were filled with a 1:1 (v:v) mix of vermiculite and sand. Soybean pots were placed in 1000 ml beakers and filled with 500 ml Jensen's medium. Alfalfa pots were placed in 600 ml beakers and filled with 200 ml Jensen's N-free medium. Jensen's medium was prepared as a 2x stock and contained per litre: $CaHPO_4$, 2.0 g; K_2HPO_4 , 0.4 g; $MgSO_4$, 0.4 g; NaCl, 0.4 g; $FeCl_3$, 0.2g; and 2 ml trace minerals. Prior to use, the solution was diluted with sterile ddH_2O. The trace mineral solution contained per 100 ml: H_3BO_3, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $MnCl_2 \cdot 4H_2O$, 0.05 g; $Na_2MoO_4 \cdot 2H_2O$, 0.05 g; Na_2EDTA, 1.0 g ; NaFe EDTA, 0.2 g; and biotin, 0.04 g. The assembly tops were covered with an aluminum foil cap and the joint between the pot and beaker sealed with a combination of aluminum foil and autoclave tape. The entire assembly was autoclaved for 4 h.

Seed sterilization and germination

Soybean [*Glycine max* (L.) Merrill cv. Bayfield or cv Maple Glen] and alfalfa (*Medicago sativa cv Iroquois*) seeds were surface sterilized as described previously (Nieuwkoop *et al.*, 1987). Seeds were surface-sterilized in 95% ethanol for 5 min, then placed in 2.5% sodium hypochlorite for 20 min. The seeds were rinsed 10 times with sterile ddH_2O over a period of 1 h, then germinated on 1.5% water agar plates in the dark at 30°C for 2-3 days. Four soybean or ten alfalfa seedlings of approximately 1-2 cm in length were planted aseptically roughly 1 cm deep in each pot. The assemblies were placed in Conviron growth chambers (Conviron Co., Winnipeg, Canada) covered with their aluminum foil lids and allowed to further grow 2 days before inoculation.

Plant growth and inoculation

Fresh cultures were grown to a density of approximately 10^8 cells·ml^{-1}. Cultures were diluted 0.2 ml in 20 ml sterile ddH_2O and added to the pots. The seedling were incubating for 28 days with a 16 h light cycle. The daytime temperature was 25°C and the night-time temperature was 20°C. The plants were watered with sterile ddH_2O when required. Plants were harvested 28 days post inoculation. Plant shoots were cut off at the root-stem boundary, dried for 4 weeks at 70°C, and then shoot dry weights were determined.

DNA manipulations

Restriction endonucleases and modifying enzymes were used according to manufacturer's protocols (Sambrook, *et al.,* 1989; Invitrogen Canada, Burlington, ON). Routine sticky-end ligations were carried out in a 20 µl volumes composed of 2:1 ratio of insert DNA to plasmid DNA in 1X T4 ligase reaction buffer with 1U of T4 DNA ligase (Invitrogen Canada, Burlington, ON). Dephosphorylation of DNA was carried out using calf intestinal alkaline phosphatase (CIAP). After restriction and purification of the DNA, 1U of CIAP was added and the reaction was incubated for 5 min. The DNA was then immediately cleaned up using QIAquick nucleotide removal kit (QIAGEN, Mississauga, Canada) according to the manufacturer's guidelines. Horizontal gel electrophoresis were conducted on 0.8% agarose gels buffered with TAE (40 mM Tris-acetate; 1 mM EDTA pH 8.0) and 0.5 µg/ml ethidium bromide (EtBr) at 85 V for ~ 1 h. DNA samples were loaded with loading buffer (0.5% bromophenol blue; and 15% Ficoll type 400). To compensate for the migration of the gel-incorporated stain towards the negative terminal, the positive buffer well was also loaded with ethidium bromide. Two molecular weight standards were used for size and concentration estimations (Invitrogen Canada, Burlington, ON). For large fragment comparisons, λ DNA *Hind*III digest was used (46 µl of 500 µg/ml λ DNA *Hind*III; 20 µl 1 M NaCl; 0.2 µl 0.5 M EDTA; 20 µl 1 M Tris-Cl pH 7.8; sterile water to a final volume of 900 µl; heated for 10 min at 65°C, cooled on ice for 10 min, then 100 µl of 10x loading buffer was added). Smaller fragments, were compared against a 100 bp DNA ladder. Gels were

visualized and photographed using the AlphaImager and the AlphaEase v5.5 software (Alpha Innotech Corp., San Leandro, CA).

DNA isolation

DNA was isolated using various commercially available kits (QIAGEN, Mississauga, Canada). Genomic DNA was isolated using DNeasy Tissue kit according to the manufacturer's instructions for Gram-negative bacteria. PCR fragments and other DNA fragments separated by agarose gel electrophoresis were excised from agarose gels using QIAEX II gel extraction kits. Plasmid DNA was extracted and purified using QIAprep Miniprep columns (QIAGEN, Mississauga, ON). Plasmid DNA was also isolated using alkaline lysis, as described by Sambrook, *et al.* (1989). Overnight cultures were grown pelleted, washed 2x with 0.85% NaCl and resuspended in 100 µl ice-cold solution I (25 mM Tris-Cl pH 8.0; 10 mM EDTA pH 8.0; and 50 mM glucose). After vortexing, 200 µl of freshly prepared solution II (1% SDS and 0.2 N NaOH) and 20 µg/ml of DNAase free pancreatic RNAase were added. Tubes were mixed gently and stored on ice for 5 min. Then 150 µl of ice-cold solution III (60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; and 28.5 ml H_2O) was added and the tubes were gently mixed. The suspension was centrifuged at 14,000 x g for 5 min and the supernatant was transferred to new tubes. The DNA solution was extracted with phenol, phenol:chloroform (1:1) and then chloroform. Two volumes of 95% ethanol were added and the tubes were placed at -80°C for 30 min. DNA was pelleted by centrifugation at 14,000 g for 15 min, washed with 70% ethanol, 95% ethanol,

dried, and dissolved in $T_{10}E_1$. DNA concentrations were estimated relative to the *Hin*dIII digested lambda DNA standard (Gibco-BRL, Life Technologies, Burlington, Ont.) using an AlphaImager (Alpha Innotech, Mississauga, Ont.) on EtBr stained gels.

RNA isolation

To avoid RNase degradation, latex gloves were worn at all times and glassware was treated by baking overnight at 180°C, lab benches and other work areas were treated with RNaseZap (Sigma), all solutions and reagents were treated with diethyl pyrocarbonate (DEPC) (0.1%) at 37°C for a minimum of 2 h or made using DEPC treated ddH_2O. Any residual DEPC that could effect purines was removed by autoclaving.

Total cellular RNA was isolated using a modified hot phenol extraction method (Chuang *et al.*,1993). An overnight culture was sub-cultured into 100 ml fresh medium and grown to an $A_{600} \sim 0.7$. The culture was then split into 50 ml volumes and 5 ml of cell stop solution (5% phenol in 100% absolute ethanol) was immediately added. The solutions were mixed vigorously by rapid inversion and then centrifuged at 3,020 × g for 10 min at 4°C. The pellets were frozen at -70°C for 2 h. The pellets were resuspended in 960 µl RNase-free water by vortexing. The samples were split into 480 µl aliquots to which an equal volume of hot phenol solution (5 parts hot phenol buffer to 1 part phenol) was added. The hot phenol buffer was composed of : 20 mM Tris, 400 mM NaCl, 40 mM EDTA, 1% SDS, and 1% β-mercaptoethanol. The tubes were vortexed thoroughly, placed in a 95°C

water bath for 1 min, then centrifuged at 14,000 × g for 10 min. The supernatant was subjected to a phenol:chloroform extraction followed by a chloroform extraction. The nucleic acids were precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol. The tubes were mixed and placed on ice for 30 min, then centrifuged at 14,000 × g for 10 min. The pellets were washed with -20°C 70% ethanol and air dried until the fringe of the pellets turned translucent, then dissolved in 85 µl RNase-free water and subjected to a DNaseI treatment to remove any traces of DNA. The RNA was treated with 5 U of DNaseI at room temperature for 20 min, then precipitated as above and resuspended in RNase-free water.

For rapid isolation of RNA, Qiagen RNeasy mini prep kits in conjunction with Qiagen Rnase-Free DNase Sets (QIAGEN, Mississauga, Canada) were used with overnight cultures according to the manufacturer's protocol for total RNA isolation.

UV mutagenesis

Cells grown in YM were harvested at an A_{600} of 0.5 to 0.7 and dispersed into glass petri dishes (100mm x 15mm) in 5ml volumes. The cells were then placed into a Stratalinker (Stratagene) and exposed to 30,000 µJ of UV energy. The cells were immediately subcultured into 1ml of fresh YM medium and incubated with agitation (200 rpm) 3 - 5 days at 30°C.

Transposon mutagenesis

Random Tn*5* transposon mutagenesis was carried out on plasmids containing a fragment of interest using a two-step process (Yarosh *et al.*, 1989). The plasmid was first introduced into the *E. coli* strain MT614 that bears a Tn*5* insertion in its chromosome. A triparental mating was then carried out to mobilize the newly mutagenized plasmid into a *polA* Sp^r mutant, MT609. Transcojugants were selected on LB containing Km, Tc (2μg/ml), Sp (50μg/ml), and thymidine. Tn*5*-B20 mutagenesis was conducted in a similar manner using the same protocol, with donor *E. coli* strain G312. Mutagenesis was also carried out using EZ::TN <KAN-2> Insertion Kit (EPICENTRE, Madison, WI) according to the manufacturer's instructions. Briefly, an insertion reaction mix consisting of: 0.2 μg target DNA, molar equivalent EZ::TN <Kan-2> transposon, EZ::TN transposase, reaction buffer and sterile ddH_2O, in a thin walled PCR tubes (Bio Plas, Inc., San Francisco, CA) and incubated for 2 h at 37°C. The reaction was stopped with the addition of EZ::TN stop solution, and then used to transform competent DH5α cells as described above.

Homogenotization

Plasmid-borne transposon insertions were recombined into the wild-type strain by plasmid incompatibility (Ruvkun and Ausubel, 1981). The transposon-carrying IncP plasmid was first introduced into the wild-type Rm1021 by tri-parental mating, then a second plasmid (pPH1JI) from the same incompatibility group (IncP) was introduced. By selecting on LB plus Nm and Gm, only

transconjugants that have homogenotized the transposon insert into their chromosome would be selected. Stable mutants were obtained by screening the homogenotes for Tc^s and curing them of the pPH1JI plasmid. Homogenotization was also carried out using an approach based on levansucrase lethality on Gram-negative bacteria (Quandt and Hynes, 1993). DNA fragments harboring transposon inserts were subcloned into pJQ200sk which carries the *sacB* gene encoding for levansucrase. The plasmid was then mobilized into wild-type strain via conjugation and double recombinants were selected on LB plus Nm and 5% sucrose.

Isolation of complementing cosmid clones

Cosmid clones were isolated via a triparental spot mating. The clone bank (Friedman *et al.*, 1982) was grown fresh from a frozen permanent in 5 ml LB plus Tc (5 μg/ml) to a A_{675} of 0.5 before being mixed in equal volumes with the mutant strain (recipient) and MT616 (mobilizer). The conjugation was performed as described above with selection on M9 succinate as well as M9 succinate plus Tc (2 μg/ml).

Southern blot hybridization

DIG-labeled probes were generated from plasmid DNA or PCR fragments using DIG DNA Labeling and Detection Kit - Nonradioactive, and DIG PCR probe synthesis Kit respectively according to the protocol guide (Boehringer Mannheim/Roche Diagnostics, Laval, QC). DNA was restricted, run on an agarose gel, photographed beside a ruler, depurinated, denatured, neutralized, transferred to

positively charged nylon membrane (Roche) by the filter paper wick method, and UV-crosslinked to the membrane (1,200 μJ [x100], UV Stratalinker 1800, Stratagene, La Jolla, CA) (Sambrook, *et al.,* 1989). A DIG-labeled probe was hybridized to the blots, which were subsequently washed and visualized by the supplier's Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) colourimetric detection method.

PCR and RT-PCR

PCR and RT-PCR were performed using thin walled PCR tubes (Bio Plas, Inc., San Francisco, CA) and a PTC-100 thermocycler (MJ Research, Watertown, MA). Standard protocol for PCR using *Taq* DNA polymerase and the provided polymerase buffer was followed (Invitrogen Canada, Burlington, ON). PCR was also conducted using the Expand High Fidelity PCR system (Roche) when increased fidelity was required. This system uses a mixture containing Taq DNA polymerase and a proofreading polymerase with 3' - 5' exonuclease activity. In general, PCR reactions (50 μL) contained 25 to 50 ng of purified genomic or plasmid DNA; 10 pmol of each primer (Table 2.2); PCR buffer (Gibco-BRL); 1.5 mM Mg^{2+} (Gibco-BRL); and 200 mM dNTPs (Roche, Laval, Que.). Template DNA was denatured at 94°C for 3 min, then 2.5 U *Taq* DNA polymerase (Gibco-BRL) was added, and the reaction was cycled 25-30 times as follows: denaturation for 1 min at 92°C; annealing for 1 min at the appropriate temperature based on the primers used; extension for 1 min at 72°C. This was followed by a final extension for 5 min at 72°C.

Table 2.2. Primers used in this study.

Primer name	Sequence (5'-3')	Tm* (°C)	Reference
27f	AGAGTTTGATCMTGGCTCAG	49	Ritchie *et al.*, 1997
1492r	TACGGYTACCTTGTTACGACTT	50	Ritchie *et al.*, 1997
BhvF1	TGTAAAACGACGGCCAGTGCCTAATACA TGCAAGTCGACCG	70	Goto *et al.*, 2000
BhvR1	CAGGAAACAGCTATGACCACTGCTGCCT CCCGTAGGAGT	71	Goto *et al.*, 2000
EZTNfor	ACCTACAACAAAGCTCTCATCAACC	73	Epicentre
EZTNrev	GCAATGTAACATCAGAGATTTTGAG	69	Epicentre
ptn5	GGAGGTCACATGGAAGTCAG	59	Wu *et al.*, 1997
pLacZ	TCCCAGTCACGACGTTGTAAAACG	63	Wu *et al.*, 1997
pVKLacZ	CCGCCACATATCCTGATCTT	57	Liu *et al.*, 2001
mdh-sucC	GCGCTTCCGGCTCTTCCTCG	59	This study
sucC-mdh	GCGCATTGCCGTCGAAGGAG	59	This study
sdhExt4	GAATGGGTGTCGGGAAGTC	59	This study
sdhExt16	CCCTGGAATCGGCGGATGAG	58	This study
sdhExt16EcoR1	CGGAATTCCGCCCTGGAATCGGCGGATG AG	74	This study
sdhExt17Kpn1	GGGGTACCCCTTGAAGATGCGGCGAGAG CG	75	This study
sdhExt18	GCGGGAAATGTCACGGATTC	54	This study
sdhExt18EcoR1	CGGAATTCCGGCGGGAAATGTCACGGAT TC	71	This study
sdhExt20	CGACGACCCAGATGACGAGC	58	This study
sdhExt20Kpn1	GGGGTACCCCCGACGACCCAGATGACGA GC	75	This study
sdhA3344f	CCATCACCACGGTCTACGG	55	This study
sdhA4706r	CCCTTGACGGTGTCGTAA	54	This study
sdhB2761r	CAGCGGATACACCTTCACG	53	This study
sdhB12223r	GCGTTTCAAATCACTTAGCAAC	57	This study
sdhB13363f	CCATCACCACGGTCTACGG	61	This study
sdhC3668f	ATGTCACGGATTCGGTTGGG	59	This study
sdhC5571f	ACGACTGGGTGAACTGGCTG	56	This study
sdhD2473r	CGTCGGGAAGACCTTGGTG	61	This study
sdhD2883r	GCATAATGGGCGAGAAAGACG	60	This study
sdhD3181f	ACCTGCTGGGCGGCATACG	63	This study
sdhD5249r	GAGAGGAATGCTGGCGACGG	58	This study
sdhD5306f	GGTCTCGGTTCGGCAAAG	53	This study

*Calculated annealing temperature

RT-PCR were performed using QIAGEN One-step RT-PCR Kit (QIAGEN, Mississauga, ON) as described by the manufacturer's protocol. The reactions contained the following: 10 µl 5x buffer; 2 µl dNTPs; 3 µl of each primer; 2 µl enzyme mix; 1.25 µl RNAase inhibitor; 1 µl total RNA; and 27.75 µl RNAase-free water. The RT-PCR reactions were carried out as follows: reverse transcription at 50°C for 30 min; reverse transcriptase inactivation and DNA polymerase activation at 95°C for 15 min. The reactions were cycled 20 times as follows: denaturation at 94°C for 45 s; annealing at 60°C (varied based on primers used) for 10 s; and extension at 72°C for 1.5 min. After cycling, a final extension at 72°C for 10 min was done.

Transcriptional start site analysis

5' Rapid amplification of cDNA ends (5'-RACE) was used to determine the transcriptional start according to the manufacturer's guidelines (Invitrogen Canada, Burlington, ON). Total RNA was isolated as described above and purified using S.N.A.P columns according to the manufacturer's protocol (Invitrogen Canada, Burlington, ON). First strand cDNA was generated using a gene-specific antisense primer (GSP1). The cDNA was tailed with terminal deoxynucleotidyl transferase (TdT) and dCTP to add a homopolymeric tail at the 3' end. PCR was then carried out to amplify the fragments using nested gene-specific primers (GSP2), which anneal 3' to GSP1, and a complementary homopolymer-containing anchor primer. The resulting replicons were directly sequenced or cloned into a sequencing vector.

DNA sequencing

DNA sequences were initially obtained from PCR fragments and plasmid DNA using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Mississauga, ON), and reactions were run on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Mississauga, ON). More recently, sequencing has been carried out by the McGill University and Genome Quebec Genome Center, where 10µl sequencing reactions were done in 96-well plates with BigDye Terminator Cycle sequencing version 2.0 and 3.0 and analyzed with a 3730XL DNA Analyzer system (PE Applied Biosystems, Mississauga, ON). Nucleotide sequences were compiled using Sequencher v. 3.0 (Gene Codes Corporation, Inc., Ann Arbor, Mich.) and compared to the nr databases of NCBI via blastn (Altschul, *et al.,* 1997). Primers were designed using Gene Tools lite version 1.0 (DoubleTwist, Inc., Oakland, CA). Sequence analyses and alignments were carried out using MacVector 7.0 (Oxford Molecular Ltd., Genetics Computer Group, Madison, WI) and the CLUSTALW algorithm (Thompson, *et al.*, 1994).

Preparation of cell-free extracts

Cultures were grown in 500ml of the appropriate media to late log phase (A_{600} ~ 1.0) and placed on ice. All of the following steps were performed on ice or at 4°C. Cultures were centrifuged at 5,860 x g for 20 min. The cells were resuspended and washed twice with cold washing buffer (20 mM Tris [pH 7.8], 1 mM $MgCl_2$). The wet weight of the pellet was determined and the pellet resuspended in 4 ml/g of sonication buffer (20 mM Tris [pH7.8], 1 mM $MgCl_2$, 10%

glycerol, and 10 mM β-mercaptoethanol). Cells were disrupted by sonication (Ultrasonics Sonifer Cell Disruptor Model W185) at 4°C for a total of 2 min in 10 s pulses followed by 50 s cooling periods. Cell debris was removed by centrifugation at 17,000 × g for 20 min. The resultant extracts were stored at -80°C until used.

Total membrane preparation

Total membranes were prepared using a slightly modified version of the method described by Niven *et al.* (1989). Cells were grown to late log phase in 250 ml liquid medium and harvested by centrifugation at 5,860 x g for 20 min. The cells were then subjected to the sonication protocol previously described in the preparation of cell extracts. The sonicated cells were centrifuged at 17,000 × g for 10 min at 4°C. 90% of the supernatant, being careful to avoid the pellet, was transferred to an ultracentrifuge tube and centrifuged at 226,000 × g for 1 h at 4°C. The pellet containing the total membrane fraction was resuspended in sonication buffer and stored at -80°C.

Protein concentration determination

Crude extracts and/or membrane preparations were thawed on ice. Protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as standard and the Bio-Rad protein assay dye (Coomassie blue).

Isocitrate dehydrogenase assay

Isocitrate dehydrogenase was assayed as described by Reeves *et al.* (1971). The assay reagents listed in Table 2.3 were added to cuvettes and the assay was initiated with the addition of isocitrate.

Table 2.3. Isocitrate dehydrogenase assay

Reagents	Stock (mM)	Volume added (µl)
Tris pH 7.8	200	100
$MnCl_2$	40	50
Na-Isocitrate	10	50
β-$NADP^+$	10	50
Cell extract (0.1mg)		as required
ddH_2O		to a volume of 1000

Isocitrate dehydrogenase activity was determined by the rate of $NADP^+$ reduction, using the extinction coefficient of NADPH at 340 nm, 6.22×10^3 $M^{-1} \cdot cm^{-1}$ (Windholtz, 1983). Specific activity ($nmoles \cdot min^{-1} \cdot mg^{-1}$) was calculated as follow

$$\text{Specific Activity} = \frac{\Delta A_{340} \text{ x } 1 \text{ ml}}{6.22 \times 10^{-3} \times 1 \text{ min} \times 0.1 \text{ mg protein}}$$

2-oxoglutarate dehydrogenase assay

2-oxoglutarate dehydrogenase was assayed according to the method described by Reed and Mukherjee (1969). The reagents listed in Table 2.4 were added to cuvettes and the assay initiated by the addition of CoA and 2-oxoglutarate.

Table 2.4. 2-oxoglutarate dehydrogenase assay

Reagents	Stock (mM)	Volume added (µl)
Potassium phosphate buffer pH 7.4	500	100
$MgCl_2$	10	100
NAD^+, trihydrate	50	40
Cystein hydrochloride	30	100
Thiamine pyrophosphate	20	10
CoA	3	20
Na-2-oxoglutarate	100	10
Cell extract (0.1mg)		as required
ddH_2O		to a volume of 1000

2-oxoglutarate dehydrogenase activity was determined by the rate of NAD^+ reduction, using the extinction coefficient of NADH at 340nm, 6.22×10^3 $M^{-1} \cdot cm^{-1}$. Specific activity ($nmoles \cdot min^{-1} \cdot mg^{-1}$) was calculated as follow:

$$\text{Specific Activity} = \frac{\Delta A_{340} \text{ x } 1 \text{ ml}}{6.22 \times 10^{-3} \times 1 \text{ min} \times 0.1 \text{ mg protein}}$$

Succinyl-CoA synthetase assay

Succinyl-CoA synthetase was assayed according to the method described by Bridger *et al.* (1969). The reagents listed in Table 2.5 were added to cuvettes and the assay initiated by the addition of CoA.

Table 2.5. Succinyl-CoA synthetase assay

Reagents	Stock (mM)	Volume added
Tris-HCl [7.2]	200	250
KCl	1000	100
$MgCl_2$	40	250
Na-succinate	40	25
ATP	10	40
CoA	10	10
Cell extract (0.1mg)		as required
ddH_2O		To a volume of 1000

Succinyl-CoA synthetase activity was measured by the increase in absorbance at 230 nm accompanying the formation of thioester bond observed with the production of succinyl-CoA from succinate. The specific activity ($nmoles \cdot min^{-1} \cdot mg^{-1}$) was calculated as follow, using the extinction coefficient for the formation of 1nmol of succinyl-CoA, 4.5×10^{-3} cm^2:

$$\text{Specific Activity} = \frac{\Delta A_{230} \text{ x } 1 \text{ ml}}{4.5 \times 10^{-3} \times 1 \text{ min} \times 0.1 \text{ mg protein}}$$

Succinate dehydogenase assay

Succinate dehydrogenase was assayed according to the method described by Veeger *et al.* (1969). The reagents listed in Table 2.6 were added to cuvettes and the assay initiated by the addition of cell or membrane extract.

Table 2.6. Succinate dehydrogenase assay

Reagent	Stock (mM)	Volume added (µl)
Potassium phosphate buffer pH 7.6	300	330
Na_2 EDTA pH 7.6	30	33
Na-Succinate	400	100
BSA	3% (w/v)	33
$K_3Fe(CN)_6$	75	67
KCN	30	33
Cell extract (0.1mg)		as required
ddH_2O		to a volume of 1000

Succinate dehydrogenase was determined by the rate of decrease of A_{455} due to the reduction of $K_3Fe(CN)_6$ (extinction coefficient of 1.5 x 10^2 $M^{-1}\cdot cm^{-1}$). Specific activity ($nmoles\cdot min^{-1}\cdot mg^{-1}$) was calculated as follow:

$$\text{Specific Activity} = \frac{\Delta A_{455} \text{ x } 1 \text{ ml}}{1.5 \times 10^{-4} \times 1 \text{ min} \times 0.1 \text{ mg protein x } 2}$$

Malate dehydrogenase assay

Malate dehydrogenase was assayed according to the method described by Englard and Siegal (1969). The reagents listed in Table 2.7 were added to cuvettes and the assay initiated by the addition of NAD^+.

Table 2.7. Malate dehydrogenase assay

Reagents	Stock (mM)	Volume added (µl)
Na-L-malate pH 7.5	850	100
Glycine-NaOH pH 10.0	500	200
NAD^+ (trihydrate)	50	25
Cell extract (0.1mg)		as required
ddH_2O		to a volume of 1000

Malate dehydrogenase was determined by the rate of NAD^+ reduction, using the extinction coefficient of NADH at 340nm, 6.22×10^3 $M^{-1} \cdot cm^{-1}$. Specific activity ($nmoles \cdot min^{-1} \cdot mg^{-1}$) was calculated as follow:

$$\text{Specific Activity} = \frac{\Delta A_{340} \text{ x } 1 \text{ ml}}{6.22 \times 10^{-3} \times 1 \text{ min} \times 0.1 \text{ mg protein}}$$

Determination of LCO production.

LCO was measured by high-pressure liquid chromatography(HPLC) (Sanjuan et al. 1992). *B. japonicum* cultures were grown in 600 ml of YM to A_{620} of

0.4 to 0.6, equal volumes were divided into four flasks (150 ml), genistein was added, and the cultures were grown for 3 days at 15 or 25°C. After the final A_{620} was measured, the cultures were extracted with 40 ml of HPLC-grade butanol by shaking for 5 min, and then left to stand overnight to separate the two phases. A 25 ml aliquot of the upper phase was collected with a glass pipette, placed in a 250 ml evaporation flask, and stored at 4°C. The volume of the extract was reduced to 2 to 3 ml by evaporation at 80°C using a Yamato RE 500 Rotary Evaporator (Yamato Scientific American Inc., Orangeburge, NY, U.S.A.) and then 4 ml of 18% acetonitrile was added. The extracts were stored, in the dark in glass tubes sealed with Parafilm at 4°C until analysis. The samples were analyzed by HPLC, using a Vydac C18 reversed-phase column (0.46 °— 25 cm, 5 μM; Vydac, Hesperia, CA, U.S.A.) and a flow rate 1.0 ml/min. An isocratic run with 18% acetonitrile (AcN/H2O; wt/wt) was conducted for at least 10 min to establish a baseline prior to each measurement. For 45 min following the loading of each sample, an isocratic elution with 18% of AcN was conducted to ensure the removal of most nonpolar light fractions and then a linear gradient of up t o 82% AcN was applied over 70 min. The retention time of the LCOs was 84 to 86 min and values were extrapolated from peak areas.

Characterization using the Biolog identification system

NEB4, NEB5, and NEB17 cells were harvested from 24 h King's Medium B (Atlas, 1995) plates for cytological staining and microscopy. The Gram reaction was determined for all strains to be identified. As all three strains were found to be

Gram positive, they were identified using Biolog GP Microplates (Biolog Inc., Hayward, Calif.), following the manufacturer's instructions. S*taphylococcus aureus* and *Bacillus cereus* were used as controls. All strains were cultured on plates of Biolog Universal Growth Medium (BUGM; Biolog Inc.) plus 1% (w/v) glucose, at 30°C for 9 h. Glucose was added to the BUGM in an attempt to limit the degree of sporulation, as directed by the manufacturer for dealing with putative *Bacillus* species. Aliquots (150 µL) of the cell suspensions were distributed into each of the 96 wells, and then the Microplates were incubated at 30°C. Colourimetric changes were measured by determining the *A*595, after 4 and 24 h, using a 3550-UV Microplate Reader (BioRad Laboratories, Mississauga, Ont.). Readings were standardized against the control well containing no carbon source. Standardized absorbance values greater than 0.1 were scored as positive. Putative identifications were made using MicroLog1 v. 3.50 software plus database (Biolog), and only similarity index (SIM) values above 0.5 were considered significant for identification purposes (Biolog).

Phylogenetic analysis

DNA sequences were compared with the nr nucleotide databases using the standard nucleotide–nucleotide BLAST (blastn) search algorithm (Altschul *et al.* 1997). Phylogenetic analysis was done using MacVector v. 7.0 (Oxford Molecular Ltd., Genetics Computer Group, Madison, Wis.). Nucleotide sequences were aligned using the CLUSTAL W algorithm (Thompson *et al.*, 1994). Phylogenetic trees were reconstructed by the neighbor-joining method (Saitou and Nei, 1987),

using the distance matrix from the alignment. Distances were calculated using both the Kimura (Kimura, 1980) and Tamura-Nei (Tamura and Nei, 1993) methods. Gaps were ignored, no gamma correction shape was specified, and for the Kimura method, the transition:transversion ratio was estimated by the algorithm (average = 1.81). Phylogenetic trees were subjected to bootstrap analysis with 1000 replications (Felsenstein, 1985). 16S rDNA sequence of the following strains (type strains, unless otherwise indicated) were obtained from GenBank (accession numbers in parentheses): *Bacillus thermoglucosidasius* (AB021197); *Bacillus stearothermophilus* (AB021196); *Bacillus weihenstephanensis* (AB021199); *Bacillus mycoides* (AB021192); *Bacillus thuringiensis* WS2625 (Z84587); *Bacillus mojavensis* (AB021191); *Bacillus vallismortis* (AB021198); *Bacillus atrophaeus* (AB021181); *Bacillus subtilis* (X60646); *Bacillus carboniphilus* (AB021182); *Bacillus psychrosaccharolyticus* (AB021195); *Bacillus marinus* (AB021190); *Bacillus flexus* (AB021185); *Bacillus niacini* (AB021194); *Bacillus megaterium* (D16273); and the out-group, the Gram-positive bacterium *Alicyclobacillus acidoterrestris* DSM 3922T (X60742).

Connecting text

The work performed in Chapter 3 was designed to examine the regulation of *Bradyrhizobium japonicum* nodulation (*nod*) genes under low root zone temperatures. *B. japonicum* mutants with altered *nod* gene induction characteristics were isolated and characterized. The majority of the experimental work, accompanying analysis, and writing of this chapter was conducted by myself, with the exceptions cited in contributions of authors (p.xx). Dr. Brian Driscoll provided critical reading of the chapter and suggested ways in which to improve the work. The published manuscript was originally written by the different authors and subsequently edited and revised by Dr. Brian Driscoll and Dr.Trevor Charles.

Chapter 3. *Bradyrhizobium japonicum* mutants with enhanced sensitivity to genistein resulting in altered *nod* gene regulation.

This chapter was adapted from the following:

Ip, H., D'Aoust, F., Begum, A.A., Zhang, H., Smith, D.L., Driscoll, B.T., and Charles ,T.C. 2001. *Bradyrhizobium japonicum* mutants with enhanced sensitivity to genistein resulting in altered *nod* gene regulation. Mol. Plant-Microbe Interac. **14**:1404-1410.

SUMMARY

Bradyrhizobium japonicum mutants with altered *nod* gene induction characteristics were isolated by screening mutants for genistein-independent *nod* gene expression. Plasmid pZB32, carrying a *nodY::lacZ* transcriptional gene fusion, was introduced into *B. japonicum* cells that had been subjected to UV mutagenesis. Ten independent transformants producing a blue color on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) but lacking genistein, indicative of constitutive expression of the *nodY::lacZ* reporter gene, were isolated. β-galactosidase activity assays revealed that while all of the 10 strains were sensitive to low concentrations of genistein, none exhibited truly constitutive *nodY::lacZ* expression in liquid culture. Soybean plants inoculated with three of the mutants were chlorotic and stunted, with shoot dry weights close to those of the

uninoculated plants, indicating the absence of nitrogen fixation. Differences in the kinetics of *nodY*::*lacZ* expression and lipochitin oligosaccharide Nod signal production suggested that the strains carried different mutations. Some of these strains may be useful in mitigating the low root zone temperature-associated delay in soybean nodulation at the northern extent of soybean cultivation.

INTRODUCTION

Soybean, a plant of subtropical origin, is now one of the most important crops cultivated in temperate North America. In the symbiotic association with *Bradyrhizobium japonicum*, soybean plants can fix nitrogen at rates of up to 200 $kg \cdot ha^{-1} \cdot year^{-1}$(Smith and Hume, 1987), eliminating the need for environmentally and economically costly nitrogen fertilizers. The generation of effective soybean root nodules is a complex and highly regulated process that requires production and exchange of specific molecular signals between the host plant and the bacterial symbiont (Stacey, 1995). One of the first interactions to take place is the secretion of isoflavones, such as genistein (Kosslak *et al.*, 1987), by the soybean plant. Genistein induces the expression of the *B. japonicum* nodulation (*nod*) genes, by stimulating either the $NodD_1$ transcriptional activator or the NodV/W two-component regulatory system, or both, resulting in induction of *nod* gene transcription (Göttfert *et al.*, 1990; Loh *et al.*, 1997; Stacey, 1995). The products of the *nod* genes mediate the synthesis of lipochitin oligosaccharide (LCO) Nod factors (Long, 1996). Nod factors are responsible for direction of the early stages of nodule

development (Sanjuan *et al.*, 1992).

Consistent with its subtropical origins, the soybean plant requires root zone temperatures (RZT) between 25°C and 30°C for optimal establishment of symbiotic activity. At the northernmost limit for production of this crop in North America, low spring soil temperatures ranging between 10°C and 15°C at 10-cm depth inhibit nodule development, and this inhibition is the major limiting factor for soybean production. At low RZT, both the accumulation of *nod* gene-inducing isoflavone compounds in the soybean root (Zhang and Smith, 1996a) and the sensitivity of the *nod* regulon to induction by these compounds (Zhang *et al.*, 1996b) are substantially reduced. Exogenous application of genistein results in short circuiting of the plant–bacterium signaling and has been proven an effective means of overcoming the adverse effects of low RZT on nodulation and N_2 fixation (Zhang and Smith, 1995, 1997). Mutants in which the *nod* genes are expressed in the absence of the plant *nod* gene induction signals might similarly be able to overcome the low RZT inhibition of nodulation, and would then be a good alternative to the direct application of genistein in the field. Our goal was thus to construct *B. japonicum* strains in which the *nod* genes are expressed in the absence of inducing molecules from the plant.

RESULTS

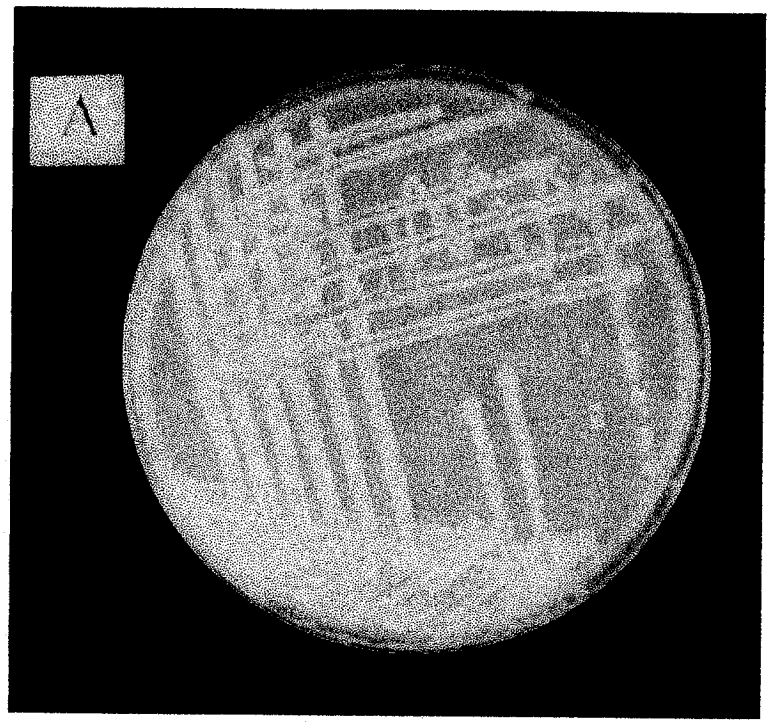
Isolation of mutants genistein-independent *B. japonicum* strains

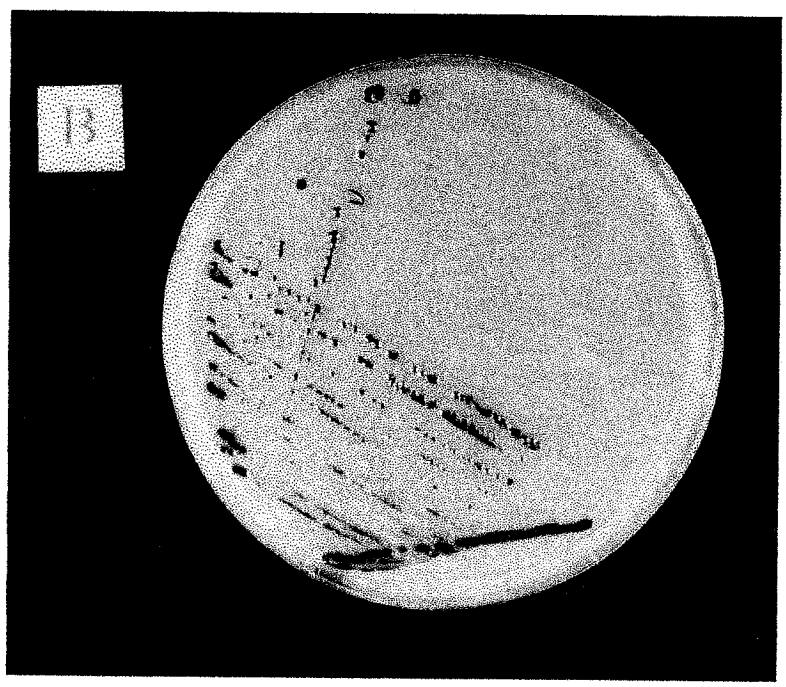
Colonies of *B. japonicum* strain ZB977, which contains the *nodY*::*lacZ*

fusion plasmid pZB32 in the USDA110 strain background, are white when grown on tryptone yeast (TY) plus Tc and X-gal, but are blue on the same medium amended with 5 μM genistein. In contrast, USDA110 (parental strain, does not carry pZB32) forms white colonies regardless of the presence or absence of genistein. USDA110 cultures were subjected to a wide range of UV energy, in order to optimize the UV mutagenesis. Using the plot of mutant frequency and total mutants versus UV energy, the dose of 30 000 μJ was chosen to generate genistein-independent strains (not shown). The mortality rate resulting from this energy dose reached upwards of 99% mortality.

To isolate *B. japonicum* mutants that constitutively expressed their *nod* genes in the absence of inducer. Plasmid pZB32 was introduced into the UV generated mutant pools by electroporation, and transformants selected on TY plus Tc and X-gal agar. Blue-pigmented transformants were streak-purified twice on the same medium. To confirm the phenotypes of the putative *nod*-constitutive mutants, the strains were cured of the *nodY*::*lacZ* reporter plasmid by screening colonies for spontaneous loss of tetracycline resistance, and then pZB32 was reintroduced by conjugation. Ten independent transconjugant colonies that appeared blue on TY plus Tc and X-gal plates were isolated (Figure 3.1). These strains, designated Bj30050 to Bj30059 (Bj30050yz to Bj30059yz if containing the reporter plasmid), thus appeared to be mutants in which the introduced *nodY*::*lacZ* fusion was expressed in the absence of genistein. All 10 mutants exhibited growth similar to the parental strain on minimal medium containing either succinate, glucose, or mannitol as sole carbon source.

Figure 3.1. Photograph of *B. japonicum* ZB977 plated on selective media (YM + 100 $\mu g \cdot ml^{-1}$ tetracycline + 80 $\mu g \cdot ml^{-1}$ X-gal) without genistein (A) and Bj30050yz plated on the same media (B). The other nine putative constitutive *nod* gene expressing mutants (Bj30051yz to Bj30059yz) demonstrated similar growth and pigmentation as Bj30050yz with some differences in terms of color intensity.


A


B

Induction of *nod* genes at different temperatures and genistein levels.

Measurements of β-galactosidase activity were carried out to quantify the expression levels of the *nodY*::*lacZ* gene fusion within the 10 mutants containing the reporter plasmid. Initial assays carried out under optimal temperature (25°C) in the absence of genistein revealed that the *nodY*::*lacZ* gene fusion was not expressed constitutively in any of the mutants (Figure 3.2). Only Bj30056yz showed any β-galactosidase activity at all, although negligible, under those conditions. However, all of the mutants were found to have much higher expression of the *nodY*::*lacZ* gene fusion, compared with the wild-type ZB977, in the presence of 5 μM genistein after a 6 h period of induction (Figure 3.2).

Prolonging the induction time with genistein to 14h resulted in slightly higher levels of *nodY* expression, but these levels were no longer different from the wild-type. Only Bj30056yz demonstrated higher than wild-type levels of *nod* expression under these conditions (Figure 3.3). Interestingly, Bj30056yz was also the only strain to show any expression in the absence of genistein. Its is widely believed that *B. japonicum nod* gene expression is regulated by cell density via NolA and $NodD_2$ (Loh *et al.*, 2001). It could be possible that these mutants have an increased sensitivity to the cell density dependent repressor as well as increased sensitivity to the isoflavone inducer. All subsequent assays were conducted with early log phase cultures (A_{600} ~0.2) to try and minimize the quorum sensing effect on *nod* expression.

To ascertain whether the genistein-sensitive mutants were able to overcome the low RZT inhibition of nodulation it was important to determine their

Figure 3.2. Expression of *nodY::lacZ* in *B. japonicum* wild-type and putative *nod*-constitutive mutant strains with (■) and without (▩) 6 h genistein induction at 25°C. Cells were induced in media containing genistein (5 μM) or methanol (control). Results are expressed in Miller Units (MU). Bars represent mean values of triplicate assays, and standard errors are indicated above the bars.

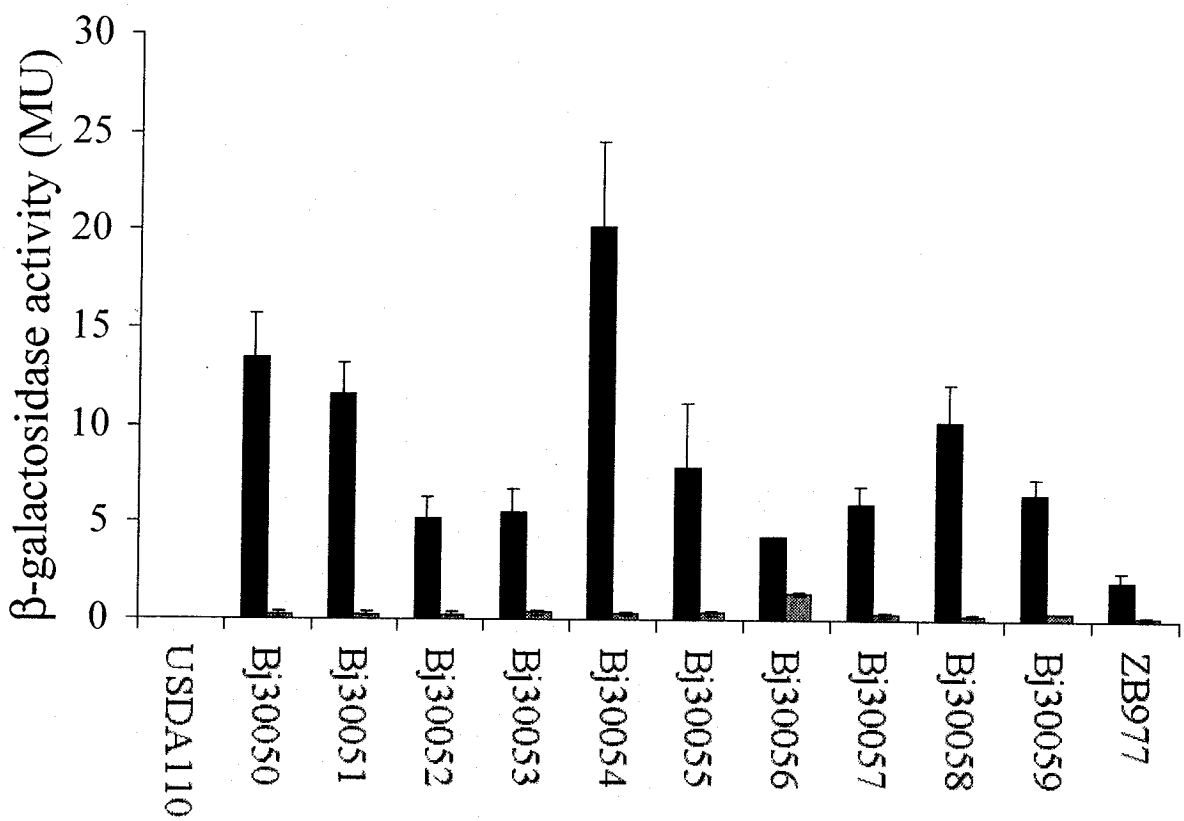
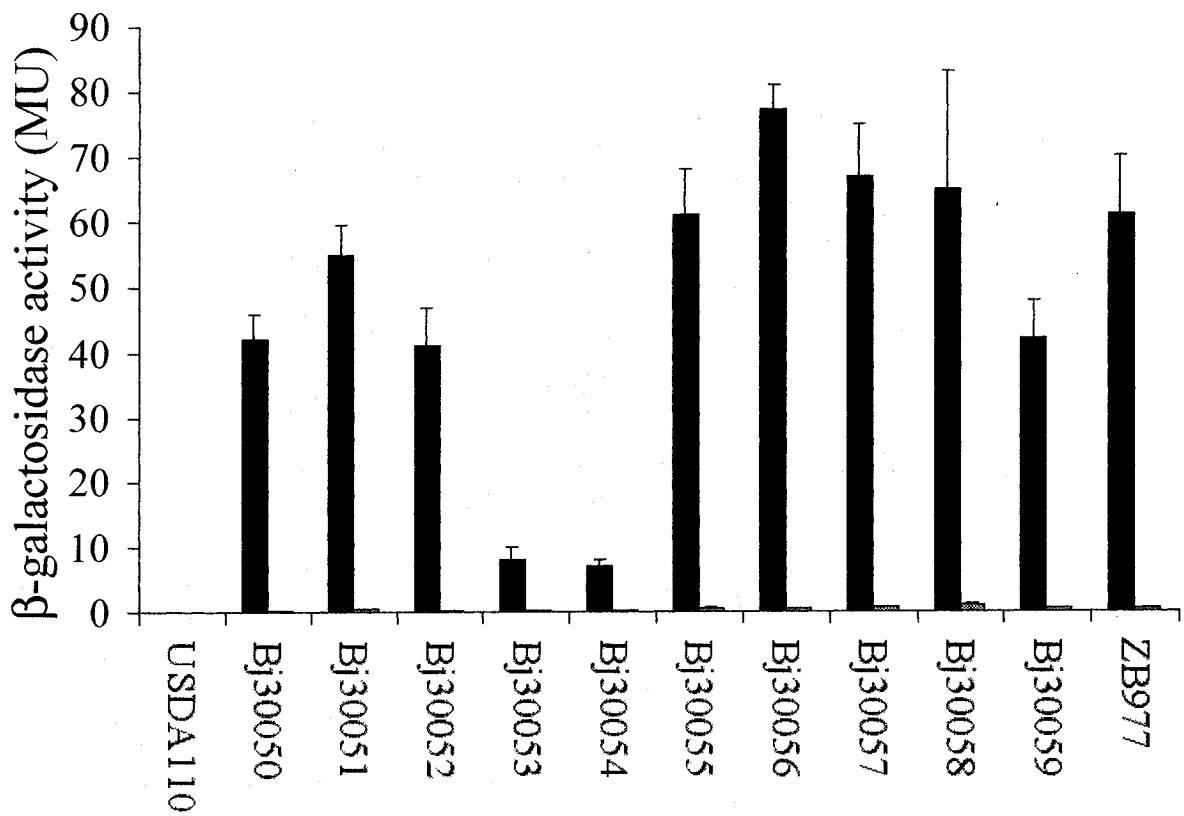

β-galactosidase activity (MU)
30
25
20
15
10
5
0
USDA110
Bj30050
Bj30051
Bj30052
Bj30053
Bj30054
Bj30055
Bj30056
Bj30057
Bj30058
Bj30059
ZB977

Figure 3.3. Expression of *nodY::lacZ* in *B. japonicum* wild-type and putative *nod*-constitutive mutant strains with (■) and without (▩) 14 h genistein induction at 25°C. Cells were induced in media containing genistein (5 μM) or methanol (control). Results are expressed in Miller Units (MU). Bars represent mean values of triplicate assays, and standard errors are indicated above the bars.


β-galactosidase activity (MU)
90
80
70
60
50
40
30
20
10
0
USDA110
Bj30050
Bj30051
Bj30052
Bj30053
Bj30054
Bj30055
Bj30056
Bj30057
Bj30058
Bj30059
ZB977

expression levels under sub-optimal conditions. Based on the initial characterization, five representative strains (Bj30050yz, Bj30053yz, Bj30056yz, Bj30057yz, and Bj30058yz) were chosen for more detailed characterization. The *nodY*::*lacZ* expression profiles of the mutants were compared with that of ZB977 under different combinations of temperature (15°C and 25°C) and genistein concentrations (0.1 μM and 5 μM). Optimal induction conditions (5 μM genistein, 25°C) were based upon previous work (Zhang *et al.*, 1996a). The suboptimal temperature (15°C) is 2°C below the critical nodulation-suppressing RZT that was previously reported (Zhang and Smith, 1994). The suboptimal genistein concentration (0.1 μM) was based on an earlier report (Smit *et al.*, 1992).

Prior to the addition of genistein, none of the five mutants showed *nodY*::*lacZ* expression levels above the basal level of the wild-type control. After 12 h under optimal conditions (25°C, 5 μM genistein), each of the mutants exhibited *nodY*::*lacZ* expression levels above that of the ZB977 wildtype strain, ranging from 129% to 155% of the ZB977 value (Figure 3.4). With 0.1 μM genistein at 25°C, the mutants exhibited *nodY*::*lacZ* expression levels ranging from four to seven times the ZB977 value under the same conditions. Induction of the *nod* genes therefore appears to be highly sensitive to genistein at 25°C in these strains.

At 15°C and 0.1 μM genistein, all five of the mutants exhibited at least two-fold greater *nodY*::*lacZ* expression than ZB977 under the same conditions, with one mutant, Bj30050yz, having 10-times the wild-type activity under these conditions, corresponding to 40% of the wild-type activity under optimal conditions (Figure 3.5). At 15°C with 5 μM genistein, *nodY*::*lacZ* expression levels in four of the

Figure 3.4. Expression of *nodY::lacZ* in *B. japonicum* wild-type and mutant strains at 25°C. Cells were induced for 12 h in YS media containing genistein at a final concentration of 5μM (■) or 0.1μM (■). Results are expressed in Miller Units (MU). Bars represent mean values of triplicate assays, and standard errors are indicated above the bars.

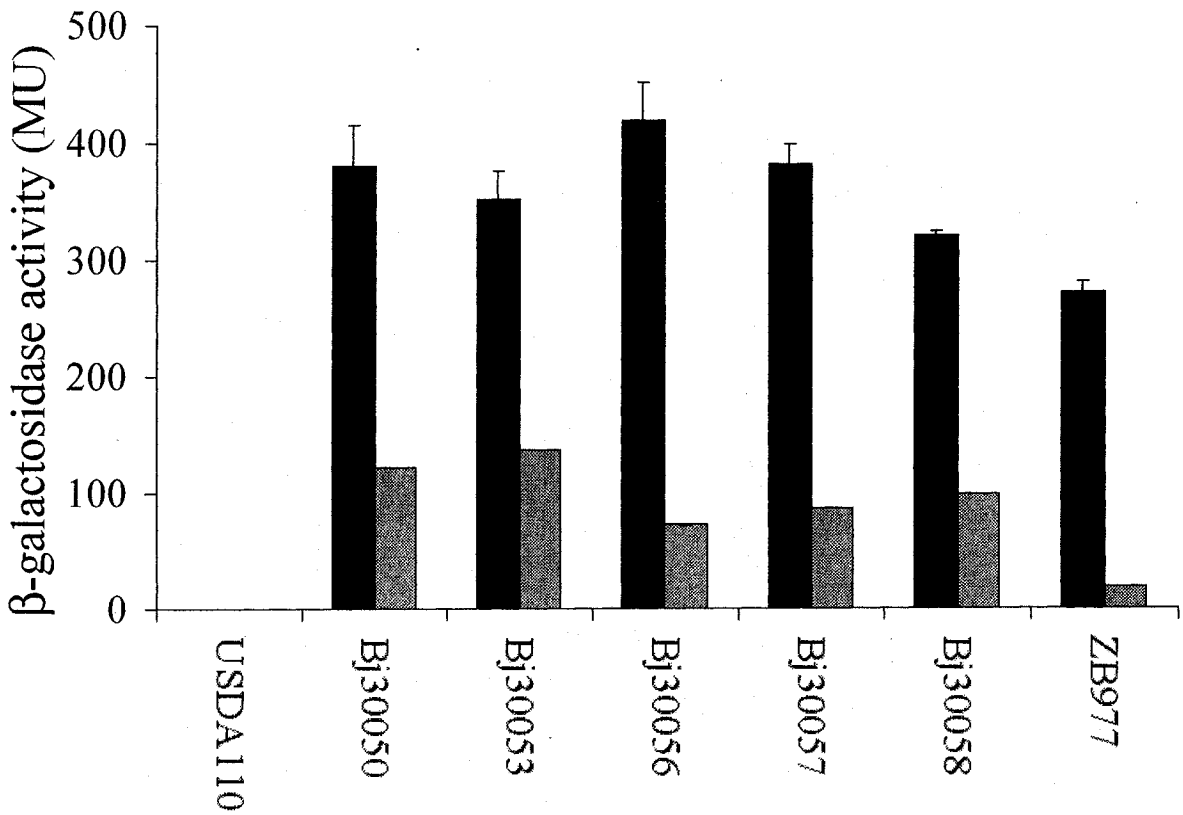
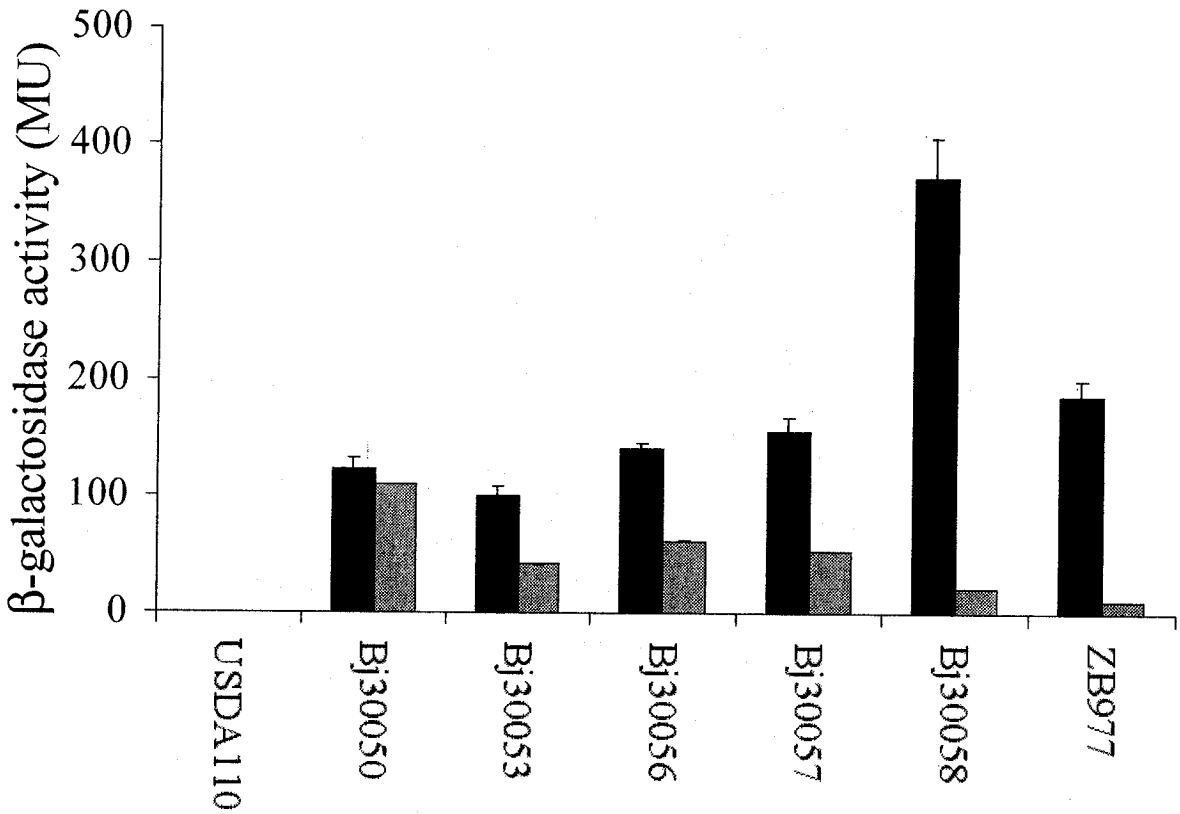

β-galactosidase activity (MU)
500
400
300
200
100
0
USDA110
Bj30050
Bj30053
Bj30056
Bj30057
Bj30058
ZB977

Figure 3.5. Expression of *nodY*::*lacZ* in *B. japonicum* wild-type and mutant strains at 15°C. Cells were induced for 12 h in YS media containing genistein at a final concentration of 5μM (■) or 0.1μM (■). Results are expressed in Miller Units (MU). Bars represent mean values of triplicate assays, and standard errors are indicated above the bars.


β-galactosidase activity (MU)
500
400
300
200
100
0
USDA110
Bj30050
Bj30053
Bj30056
Bj30057
Bj30058
ZB977

five mutant strains was reduced to 30-50% of the levels for the same strains at 25°C with 5 μM genistein (Figure 3.4 and 3.5). In fact, at the higher genistein concentration, low temperature had a greater inhibitory effect on *nodY::lacZ* expression in these four mutants than it did on the wild-type strain. Only strain Bj30058yz had a higher level of *nodY::lacZ* expression than that of ZB977 under these conditions; indeed, expression in strain Bj30058yz with 5 μM genistein was higher at 15°C than it was at 25°C.

Symbiotic phenotypes of the mutants.

Soybean nodulation assays were performed using strains cured of pZB32 to determine the symbiotic phenotype of the mutants (Table 3.1). All 10 mutants were able to induce nodules on soybeans at 25°C. There was considerable variation, however, in the apparent levels of N_2 fixation. Plants in association with three of the mutants (Bj30051, Bj30056, and Bj30057) appeared chlorotic and stunted (Fix^-; Figure 3.6). Plants inoculated with the other seven mutants appeared similar to plants inoculated with the wild-type strain.

LCO production by the mutant strains.

In the absence of genistein, there was no detectable LCO production by any of the five representative mutant strains or the wild-type (data not shown). When 0.1 μM genistein was present, all five mutant strains were found to produce at least twice as much LCO as USDA110 at the corresponding temperature (Table 3.2). At 25°C, Bj30058 produced nearly five times as much LCO as the wild-type. At 15°C,

Table 3.1. Shoot dry weight values of soybean plants inoculated with *Bradyrhizobium japonicum* mutants.

Inoculated strain	SDW[a]	%Wild type	Symbiotic phenotype
USDA110	1.86 ± 0.08	100	Fix^+
Uninoculated	1.19 ± 0.04	64	Fix^-
Bj30050	1.73 ± 0.08	93	Fix^+
Bj30051	0.99 ± 0.04	53	Fix^-
Bj30052	1.69 ± 0.12	91	Fix^+
Bj30053	1.92 ± 0.13	103	Fix^+
Bj30054	1.92 ± 0.07	103	Fix^+
Bj30055	1.96 ± 0.10	105	Fix+
Bj30056	1.14 ± 0.08	61	Fix^-
Bj30057	1.22 ± 0.01	66	Fix^+
Bj50058	1.39 ± 0.07	75	$Fix^{+/-}$
Bj30059	1.92 ± 0.13	103	Fix^+

[a]Shoot dry weight (SDW) values are presented as mean mg per plant ± standard error of the mean. Eight replicas were done for USDA110 and the uninoculated plants, and four replicas were done for the rest. Data are of one experiment and were consistent upon replication.

[b]The ability to fix nitrogen (Fix) was determined by evaluation of the SDW and appearance of plants. Fix^+ plants appeared green and healthy (similar to those inoculated with USDA110), Fix^- plants appeared chlorotic and stunted (similar to plants not inoculated with *B. japonicum* (uninoculated controls)), and plants that were intermediate were scored as $Fix^{+/-}$.

Figure 3.6. Photograph of soybean [*Glycine max* (L.) Merr.] cv. Maple Glen plants inoculated with mutants (Bj30054, Bj30055, Bj30056, Bj30059) or uninoculated (Control). Wild-type USDA 110 is not shown but growth and appearance was similar to Bj30055. Soybean were grown for 28 days post-inoculation.

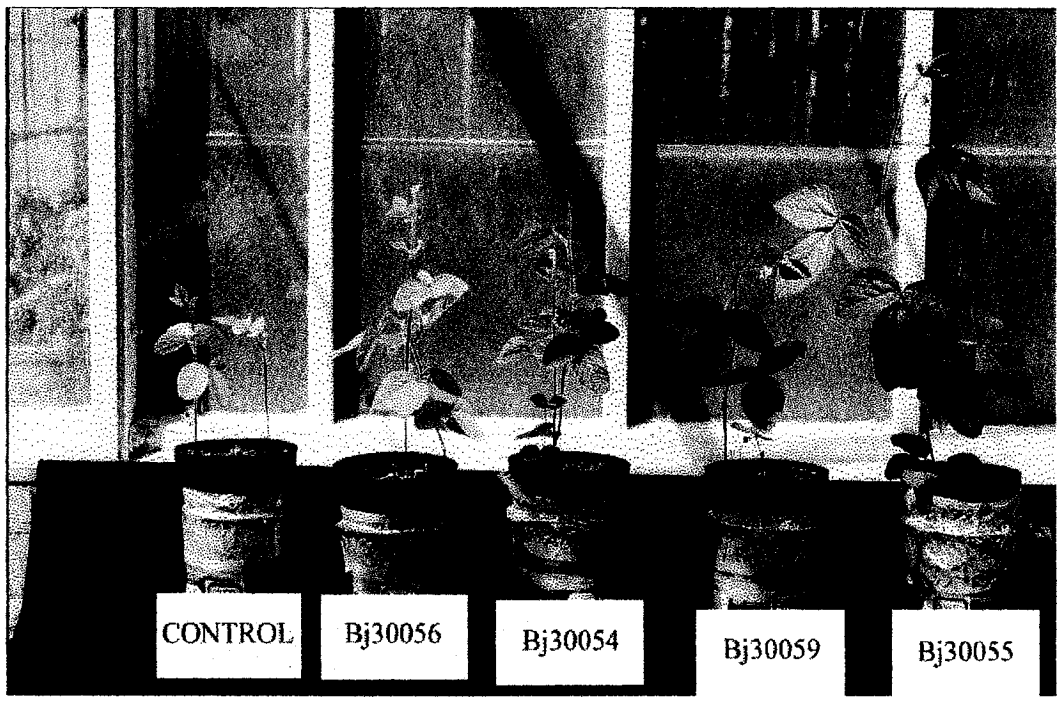

CONTROL
Bj30056
Bj30054
Bj30059
Bj30055

Table 3.2. Production of lipochitooligosaccharide (LCO) by *Bradyrhizobium japonicum* strains.

Strain	25°C		15°C	
	LCO[a]	% Wild type[b]	LCO	% Wild type
USDA110	1,650	100	979	59
Bj30050	3,640	221	2,250	136
Bj30053	5,370	325	4,440	269
Bj30056	6,840	415	6,230	376
Bj30057	6,380	387	5,670	344
Bj30058	8,140	493	5,640	342

[a]Lipochitooligosaccharide (LCO) production ($\times 10^{-12}$ μg· $cell^{-1}$) from cultures induced with 0.1 μM of genistein. LCO production expressed as mean value of triplicate measurements. The standard deviation values were within 10% of the mean of all determinations.

[b]Relative values expressed as a percentage of the USDA110 value at 25°C.

Bj30056, Bj30057, and Bj30058 each produced more than five times the level of the wild-type. This is an intriguing result, considering Bj30056 and Bj30057 were found to have a Fix^- phenotype on soybean. Together with the results from the β-galactosidase assays, these results indicate that the mutants are, in general, more sensitive to genistein with respect to *nod* gene induction and subsequent production of LCO.

DISCUSSION

Earlier studies had shown that induction of the *nodY* operon in strain USDA110 by moderate levels of genistein was reduced at lower temperatures (Zhang *et al.*, 1996a). Higher genistein levels (20 μM) resulted in substantially increased levels of *nod* gene expression at the low temperatures. In a subsequent paper (Zhang and Smith, 1996b), the authors proposed that nodulation delays at low RZT were due, in part, to the reduced sensitivity of *B. japonicum* to plant-derived isoflavone signals, and, in part, to the decreased production of isoflavone signals by the plant. This led to the idea that genetic uncoupling of the *nod* gene-encoded LCO production from the requirement for activation by isoflavone might circumvent the low RZT inhibition of nodulation.

The initial objective of this work was to develop constitutive *nod* gene expression mutants by UV mutagenesis, in order to overcome the delay of nodulation of soybean caused by suboptimal RZTs early in spring. Intriguingly, each of the characterized mutants turned out to be genistein ultrasensitive but not

genistein independent when *nodY*::*lacZ* expression was assayed in aqueous culture. Consistent with this, no detectable levels of LCO Nod factor were produced by the mutants without genistein addition and they produced higher levels of Nod factor whenever genistein was added to the culture.

Perhaps the mutants were ultrasensitive to trace amounts of inducing compounds present in the agar in the solid media. Other aromatic compounds not found in soybean roots, such as xanthone molecules, were previously found to be inducers of the *nod* genes in *B. japonicum* (Cunningham *et al.*, 1991; Yuen *et al.*, 1995). Because the mutants required much lower levels of genistein for *nodY* operon induction at both optimal and low temperatures and exhibited higher rates of *nod* gene activation, we concluded that the mutants have enhanced sensitivity to genistein for *nod* gene induction.

The five mutants that were characterized in more detail can be classified according to kinetics of *nodY*::*lacZ* induction and symbiotic phenotype. That each mutant had a distinct *nodY*::*lacZ* expression pattern reflected that they were independent and distinct isolates. However, they can be roughly divided into three groups. From initial induction rates of *nodY*::*lacZ* expression, it was apparent that Bj30050 and Bj30053 respond similarly to the four different induction conditions. The effect of low temperature on *nodY*::*lacZ* induction was greater than that of suboptimal genistein level (Figure 3.4 and 3.5). Similarly, Bj30056 and Bj30057 can be grouped together as another class (Figure 3.4 and 3.5, Table 3.2). In these two strains, *nod* expression of low temperature was similar to that of suboptimal genistein level, and the two effects were clearly additive, as they are in the wild-type

strain. The LCO production levels were also similar under the different conditions (Table 3.2). Moreover, soybean nodules formed by these strains were both Fix$^-$. The fifth mutant strain, Bj30058, does not belong to either of the first two classes. Its *nodY*::*lacZ* expression phenotype was distinct from the other four mutants. Bj30058 was far less sensitive to induction by suboptimal levels of genistein, and the adverse effect of low temperature were only observed at the low genistein concentration.

It was initially predicted that some or all of these mutants might have Fix$^-$ phenotypes due to constitutive *nod* gene expression within the bacteroid. Elevated levels of expression of the *Rhizobium leguminosarum* bv. *viciae nod* genes in nodules, whether from a multicopy plasmid (Knight *et al.*, 1986) or as the result of *nodD* mutant alleles that cause enhanced sensitivity to inducer (Burn *et al.*, 1987), resulted in a Fix$^-$ phenotype. Suppression of *nod* gene expression in wild-type bacteroids (Schlaman *et al.*, 1991) has also been demonstrated. In *B. japonicum*, severe inhibition of *nodD*1 and *nodY* gene expression (up to 90%) occurred when specific organic acids such as L-malate and fumarate were present in the culture (Yuen and Stacey, 1996). Since these organic acids are recognized as the primary carbon sources by the plant to the bacteroids, such results suggested a mechanism by which *nod* gene expression is inhibited in bacteroids. Although none of our mutants exhibited constitutive *nod* gene expression, three (Bj30051, Bj30056, and Bj30057) out of ten were Fix$^-$. Considering the nature of UV mutagenesis, the possibility of more than one mutation contributing to the observed phenotype must not be discounted.

The underlying mechanism of *nod* gene regulation has been recognized as complex. Our collection of characterized novel mutants covering a spectrum of *nod* induction will be a useful tool for further studies on the fundamental mechanisms of *nod* gene regulation and the basis for disruption of nodulation at suboptimal RZT. Investigation of the genetic basis of the mutant phenotypes will contribute toward the understanding of the gene regulatory system. Of particular interest will be determination of whether there are any alterations in expression or activation of the regulatory *nod* genes (e.g., *nodD1*, *nodVW*) in these mutants, perhaps akin to the *R. leguminosarum* bv. *viciae nodD* class IV mutants described by Burn and associates (1987, 1989). Such studies should lead to resolution of the mechanistic nature of the enhanced sensitivity and provide further insight into the sophisticated *nod* gene regulation during symbiosis. Finally, one or more of the mutant strains might prove to be of practical use and commercial value as an inoculant that is not subject to the low RZT nodulation inhibition effect.

Connecting text

The previous Chapter (3) discussed the isolation and characterization of *B. japonicum* mutants with altered *nod* gene expression. The next step would have been to genetically characterize the mutations responsible for the phenotype observed. For reasons that are explained below we believed this to be unfeasible. We therefore decided to focus our attention on the regulation of another important aspect of symbiotic N_2-fixation shown to be effected by low RZT, carbon metabolism. The work performed in Chapter 4 was designed to gain a better understanding of the role and regulation of SDH in *S. meliloti*. The *sdhCDAB* genes were shown to be co-transcribed, the *sdhC* transcription start site was mapped, and *lacZ* gene fusion studies of the regulation of the *sdh* operon by carbon source were done. The experimental work, accompanying analysis, and writing of this chapter was conducted by myself. Dr. Brian Driscoll provided critical reading of the chapter and suggested ways in which to improve the work.

Chapter 4. Characterization of the *Sinorhizobium meliloti sdh* operon

SUMMARY

The genes encoding SDH were shown by RT-PCR to be arranged in an operon with the gene order *sdhCDAB*. The transcriptional start site and putative promoter region were determined by 5'-RACE analysis. Transcriptional *lacZ* fusions to the *sdh* promoter region indicated that regulation of the operon was effected by carbon source and independent of growth phase. The expression of the *sdh* operon was lowest in cells grown with glucose or pyruvate, and highest in cells grown with acetate or arabinose as sole carbon sources.

INTRODUCTION

We previously isolated and characterized *B. japonicum* mutants with altered *nod* gene expression. These mutants were found to have increased sensitivity to genistein that translated into higher LCO production under low RZT. The next step would have been to genetically characterize the mutations responsible for the phenotype observed. However due to the nature of the mutation (random UV mutagenesis) and the complexity of *nod* gene regulation in *B. japonicum* it would have been necessary to sequence the entire collection of genes involved in *nod* regulation for several strains, with the possibility of the end result not providing much insight.

We therefore decided to focus our attention on the regulation of another important aspect of symbiotic N_2-fixation shown to be effected by low RZT, carbon metabolism. Symbiotic N_2-fixation is an extremely energy-expensive reaction that requires the bacterium to produce large amounts of energy and reducing power under oxygen-limited conditions. It has been shown that rhizobia most likely generate these requirements primarily thought the oxidation of C_4-dicarboxylic acids via the TCA cycle (for review see Vance and Heichel, 1991, Dunn, 1998; Poole and Allaway, 2000).

SDH is the TCA cycle enzyme complex responsible for the oxidation of succinate to fumarate coupled with the reduction of ubiquinone to ubiquinol. Most of what is known about this enzyme complex comes from studies conducted with *E. coli* and *B. subtilis* (Ackrell *et al.*, 1992). SDH activity has been shown to decrease under suboptimal temperature in rhizobia. Cold-sensitive *Rhizobium* mutants demonstrated decreased SDH activity under suboptimal temperatures compared to cold-acclimated rhizobial strains (Sardesai and Babu, 2000). The present study was undertaken to characterize the *sdh* operon in *S. meliloti*, with the general objective of increasing our understanding of basic gene regulation in this system.

RESULTS

Transcription analysis of the *sdh* operon using RT-PCR

Examination of the complete *S. meliloti* genome nucleotide sequence (Galibert *et al.*, 2000; Galibert *et al.*, 2001) revealed that the *sdh* genes are found in

a cluster with the order *sdhCDAB*. A putative rho-independent transcriptional terminator sequence was found 37 bp immediately downstream of *sdhB*. The genes were observed to be located on the chromosome in very close proximity to one another suggesting they form an operon. The sequential gaps between the *sdhCDAB* genes were found to be 10 bp, 7 bp, and 18 bp respectively.

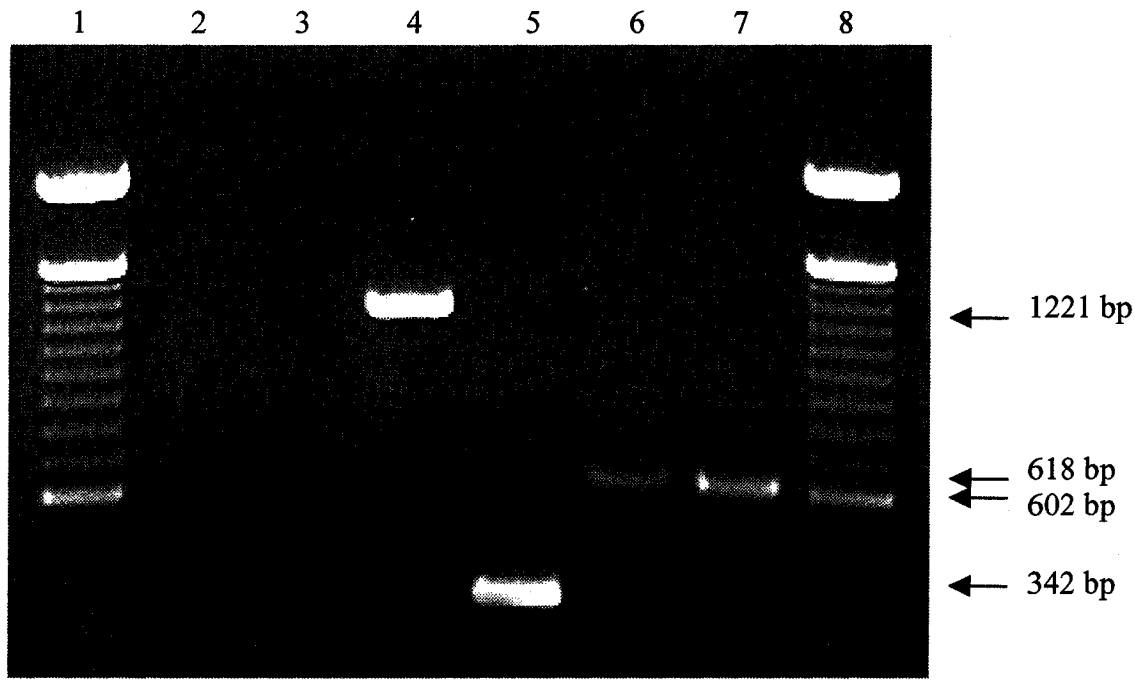
RT-PCR was performed to determine if *sdh* genes were cotranscribed, and thus formed an operon (Figure 4.1). Total RNA was isolated from a log-phase culture of *S. meliloti* grown under optimal conditions and used to generate cDNA. The cDNA was then amplified with primers designed to demonstrate cotranscription (Table 2.2; Figure 4.1). Transcripts were amplified for all three intergenic regions demonstrating that all four genes were cotranscribed as a single polycistronic mRNA. Bands were visible with predicted sizes of 342 bp, 618 bp, and 602 bp corresponding to the predicted product size generated by the *sdhC-sdhD*, *sdhD-sdhA*, *sdhA-sdhB* gene spanning regions (Figure 4.1). The negative controls consisting of RT-PCR reactions without the addition of RNA template or reverse transcriptase, yielded no visible products. A positive control consisting of amplification of the transcript spanning the *mdh* to *sucC* region as previously reported, was successful (Dymov *et al.*, 2004).

Identification of the *sdhC* promoter

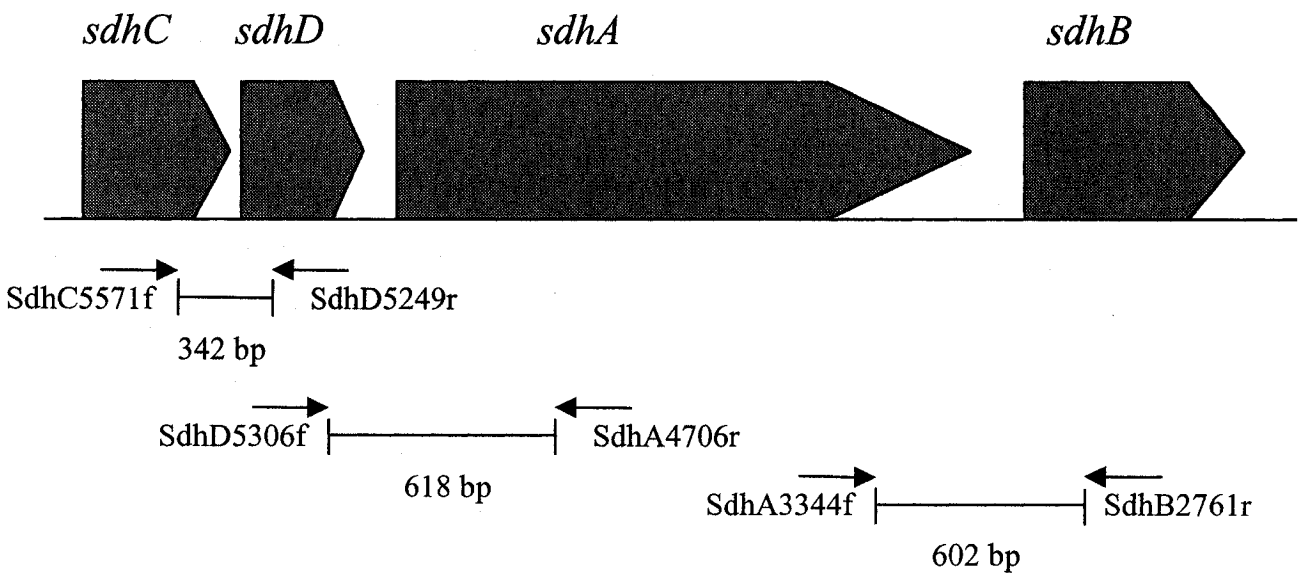
Initially, non-radioactive DIG-labeled primer extension was evaluated as a method to determine transcriptional start sites, however, we obtained inconsistent results for the *sdhC* promoter (data not shown). The low delectability of the reverse

Figure 4.1. **A.** Co-transcription analysis of the *sdhCDAB* operon using RT-PCR. Agarose gel electrophoresis of RT-PCR products that span the intergenic regions of the *sdhCDAB* operon. Lanes 1 and 9, 100 bp ladder; lane 2, no RNA; lane 3, no reverse transcriptase; lane 4, *mdh-sucC* (positive control); lane 5, *sdhC-D*; lane 6, *sdhD-A*; lane 7 *sdhA-B*. **B.** Schematic representation (not to scale) of the primer locations and corresponding gene spanning regions.

A.


1
2
3
4
5
6
7
8
1221 bp
618 bp
602 bp
342 bp

B.


sdhC
sdhD
sdhA
sdhB
SdhC5571f
SdhD5249r
342 bp
SdhD5306f
SdhA4706r
618 bp
SdhA3344f
SdhB2761r
602 bp

transcriptase product made it very difficult to determine the transcriptional start site. Therefore, the transcriptional start site of *sdhCDAB* operon was determined using the 5'-rapid amplification of cDNA ends (5'-RACE) technique. This method has been successfully used to determine the trasncriptional start site and promoter regions of other genes in *S. meliloti* (Venkova-Canova *et al.*, 2004). To ensure reproducibility and increase fidelity, two different 5'-RACE reactions using different sets of primers were used to generate both the cDNA and for sequencing.

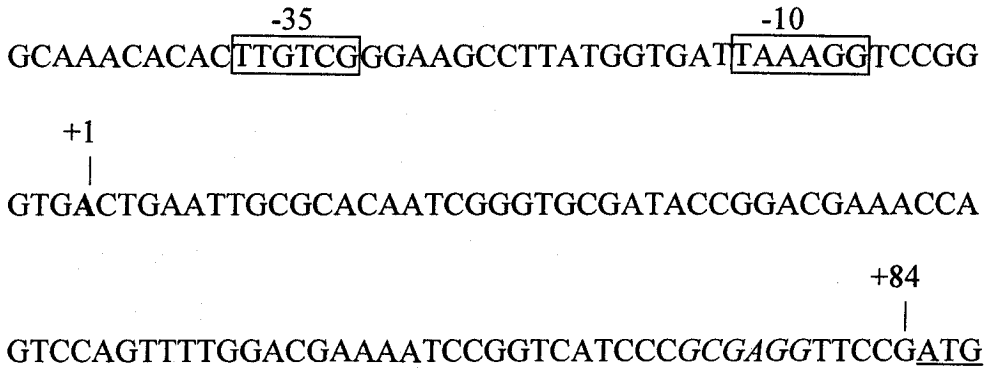
In the first 5'-RACE reaction, primer sdhD2883r was used as GSP1 to generate cDNA. The synthesized cDNA was then amplified with primers sdhD5249r as GSP2 along with the Abridged Anchor Primer (AAP) provided with the kit. A second 5'-RACE reaction was performed using primer sdhD5249r as GSP1 for cDNA synthesis and primers sdhExt20 as GSP2 combined with the AAP for cDNA amplification. The cDNA was sequenced using either sdhD5249r and/or sdhExt20 as sequencing primers. Results from both independent 5'-RACE reactions demonstrated the transcriptional start site to begin 84 bp before the predicted start codon of *sdhC* at nucleotide position 3334343 of the *S. meliloti* chromosome (Figure 4.2).

Effects of carbon source on expression of the *sdh* promoter region

Transcriptional gene fusions were constructed to investigate *sdhC* regulation, especially the role of carbon source on expression. Transcriptional fusions were generated using the suicide vector pVIK112 which encodes translational termination codons in all three reading frames followed by a multiple

Figure 4.2. Promoter sequence of the *sdhCDAB* operon in *S. meliloti*. **A.** The transcriptional start site as determined by 5'-RACE analysis in bold is indicated as +1 and the ATG translational start codon of *sdhC* gene is underlined. The putative -10 and -35 promoter regions are enclosed in boxes and ribosomal binding site is italicized. **B.** Sequence comparison of the putative *S. meliloti sdh* promoter with other *S. meliloti* promoters (Steven, 2003) and *sdh* promoters of other organisms (Wilde and Guest, 1986; Melin *et al.,* 1987; Heinzen *et al.*, 1995; Westenberg and Gardiol, 1999). The gap number shown between the -10 and -35 promoter regions represents the distance between the promoter regions in nucleotide.

A.



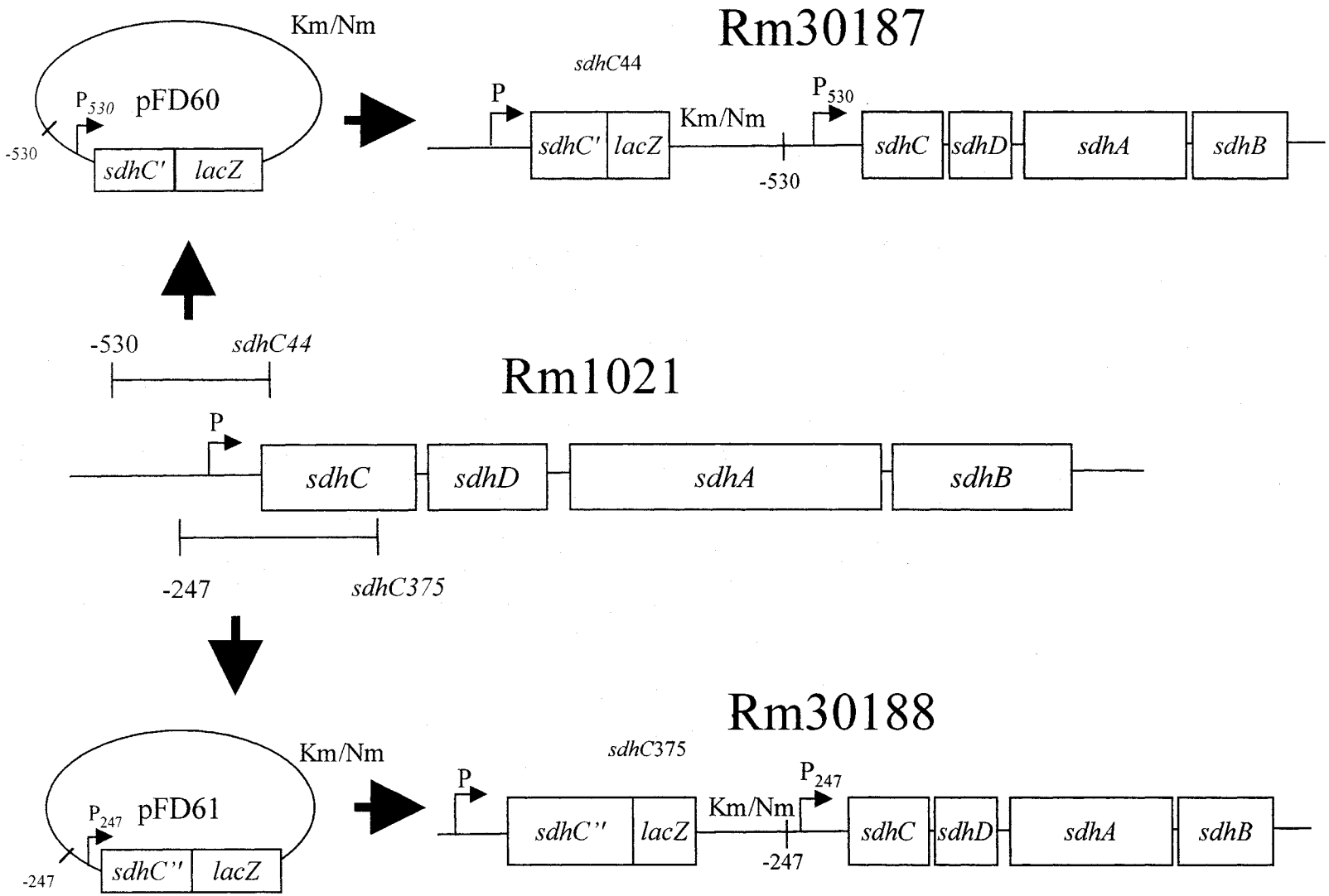
B.

Organism	gene	-35	-gap-	-10
S. meliloti	*sdhC*	TTGTCG	-17-	TAAAGG
B.japonicum	*sdhC*	TTGCGC	-17-	TAAATA
E. coli	*sdhC*	TTGTAA	-17-	TATACT
B. subtilis	*sdhC*	TTGACG	-17-	TAAAAT
Coxiella burnetii	*sdhC*	TTGCAT	-18-	TATAAT
S. meliloti	*mdh*	TTGATA	-16-	TTAAGG
S. meliloti	*pckA*	TTGTCT	-17-	TATGTT

cloning site and downstream ribosome binding site and *lacZ* gene (Kalogeraski and Winans, 1997). Cloning DNA into the MCS of pVIK112 results in the generation of a transcriptional fusion between the inserted DNA and *lacZ*. In addition, the plasmid contains the vegetative origin of R6K, but lack the R6K *pir* gene, and therefore fails to replicate in strains lacking *pir*. Hence, the entire plasmid can be integrated into the chromosome of bacterial strains lacking *pir* by homologous recombination. As the intergrations are a result of single recombination events, the resulting strain contains a fusion to the inserted DNA and a complete copy of the inserted DNA *in cis*. Therefore by constructing plasmids that contained fragments of the *sdhC* gene with different length of the promoter sequence, the importance of the promoter region could be assessed.

Plasmids used to generate the transcription gene fusions were designated pFD60 and pFD61 and differed in the amount of 5' promoter leader sequence and distance from the promoter to the *lacZ* gene (Figure 4.3). Plasmid pFD60 consisted of *S. meliloti* DNA amplified using primers sdhExt16EcoR1 and sdhExt17Kpn1 cloned into the *Eco*RI and *Kpn*I restriction sites of pVIK112. Plasmid pFD61 was generated from DNA amplified using primers sdhExt18EcoR1 and sdhExt20Kpn1 and cloned in a similar manner. The plasmids were transferred to the Lac$^-$ *S. meliloti* strain RmG212 by conjugation, and transconjugants (Rm30187 and Rm30188) were selected on LB containing Sm+Nm. Confirmation that the transconjugants were RmG212 X pVIK112 cointegrates was conducted by PCR using primers specific for pVIK112 (pVKlacZ) and pFD60 (sdhExt16) or pFD61 (sdhExt18). The expected PCR fragment would only be amplified in

Figure 4.3. Schematic representation of the strategy used to construct *S. meliloti* strains Rm30187 and Rm30188 containing chromosomal transcriptional fusions to the *sdh* promoter region. PCR fragments were amplified and cloned into pVIK112 resulting in plasmids pFD60 and pFD61 as indicated by the vertical arrows. The insert in pFD60 extended from -530 bp upstream to +44 bp downstream of the putative *sdhC* start codon, whereas the insert in pFD61 spanned the region from -247 bp upstream to +375 bp downstream of the putative *sdhC* start codon. These plasmids were recombined by single cross-over events into RmG212 generating strains Rm30187 and Rm30188, respectively, as indicated by horizontal arrows.


Km/Nm
pFD60
P_{530}
-530
sdhC'
lacZ
Rm30187
sdhC44
P
P_{530}
Km/Nm
-530
sdhC'
lacZ
sdhC
sdhD
sdhA
sdhB
-530
sdhC44
Rm1021
P
sdhC
sdhD
sdhA
sdhB
-247
sdhC375
Km/Nm
pFD61
P_{247}
-247
sdhC''
lacZ
Rm30188
sdhC375
P
P_{247}
Km/Nm
sdhC''
lacZ
sdhC
sdhD
sdhA
sdhB
-247

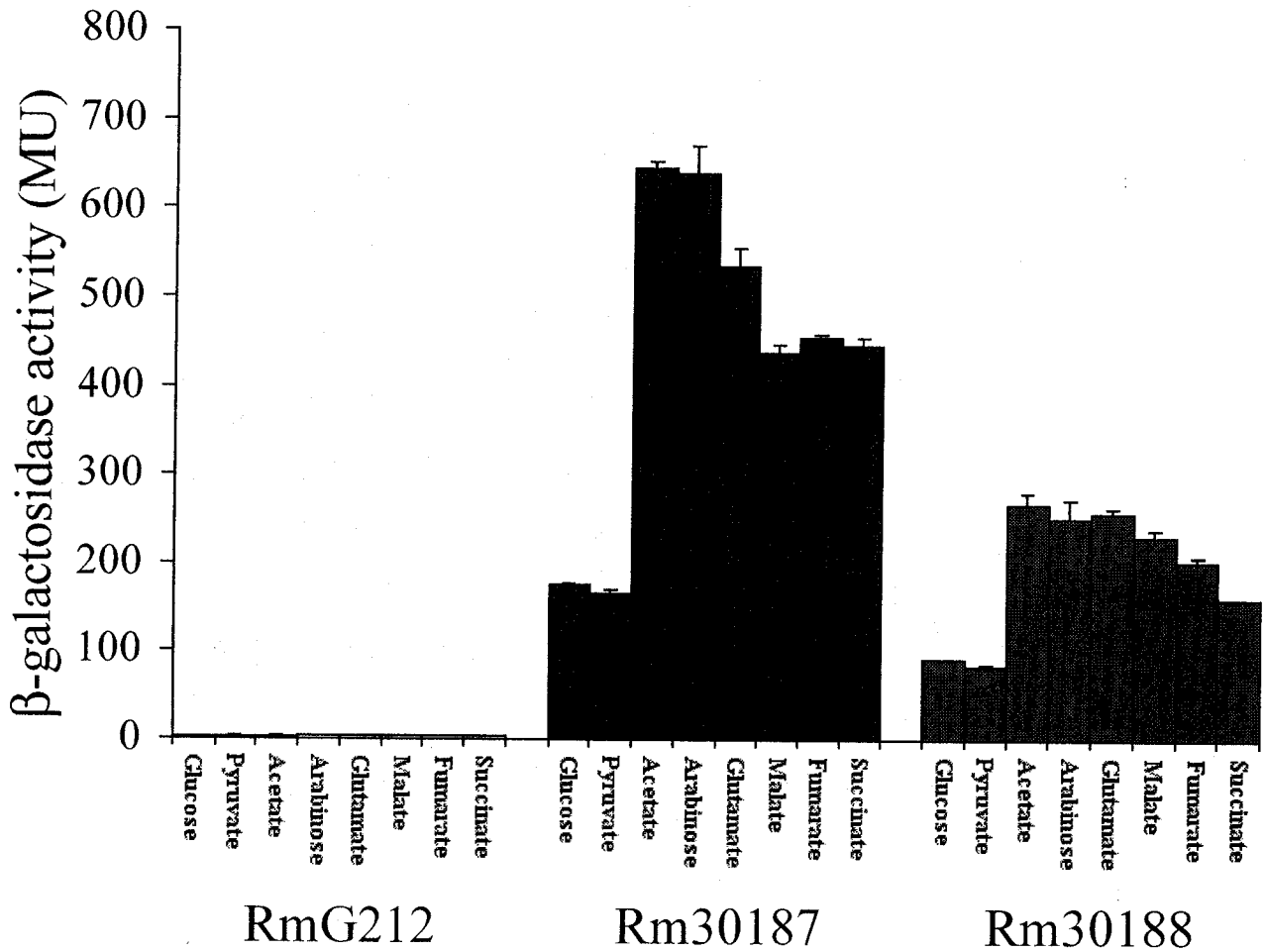
transconjugants (data not shown).

The integration sites within the cointegrate transconjugants were confirmed by sequencing using primer pVKlacZ. Rm30187 (*sdh44::lacZ*) contained in order: the wild-type *S. meliloti sdhC* promoter, a truncated *sdhC* gene fused to *lacZ*, pVIK112 vector, the cloned *S. meliloti* fragment extending from -530 bp upstream to +44 bp downstream of *sdhC* start codon (P_{530}) followed by the rest of the wild-type *sdh* operon. Rm30188 *(sdh375::lacZ)* contained in order: the wild-type *S. meliloti sdhC* promoter, a truncated *sdhC* gene fused to *lacZ*, pVIK112 vector, the cloned *S. meliloti* fragment extending from -247 bp upstream to +375 bp downstream of *sdhC* start codon (P_{247}) followed by the rest of the wild-type *sdh* operon. (Figure 4.3).

Cointegrate transconjugants were only obtained when the putative *sdhC* promoter was present on the fragment cloned into pVIK112. All attempts of generating transcriptional fusions with inserted DNA that did not contain the putative *sdhC* promoter region cloned into pVIK112 were unsuccessful, indicating the importance of the *sdhC* promoter for cell viability. Taking into account that lethality is very difficult to demonstrate, it is possible that the lack of transconjugants could be explained differently. The cells could have been viable but had an extremely slow growth phenotype. On the other hand, since cointegrate transconjugants were generated form both pFD60 and pFD61, the difference in 5' promoter leader sequence between P_{530} and P_{247} are not important for cell viability.

The cointegrate strains (Rm30187 and Rm30188) carrying *sdhC::lacZ* transcriptional fusions were then used to determine the influence of carbon source

Figure 4.4. The effect of carbon source on the expression of the *sdh* promoter region of *S. meliloti*. Strains used in this study: (□) RmG212, (■) Rm30187 (RmG212 *sdh44::lacZ*), and (■) Rm30188 (RmG212 *sdh375::lacZ*). Cells were grown in M9 supplemented with 15mM carbon source and assayed at late log phase. The bars represent β-galactosidase activity in Miller units (MU), values are the means of triplicate assays with standard errors indicated above the bars.


β-galactosidase activity (MU)
800
700
600
500
400
300
200
100
0
Glucose
Pyruvate
Acetate
Arabinose
Glutamate
Malate
Fumarate
Succinate
RmG212
Rm30187
Rm30188

on *sdh* expression (Figure 4.4). Cultures were grown to late log-phase in M9 media supplemented with the various carbon sources and then assayed for β-galactosidase activity. As expected, expression levels detected with Rm30188 were consistently lower than those of strain Rm30187 due to the distance between the promoter and *lacZ* gene. The same phenomenon was observed with *mdh* transcriptional fusions (Steven, 2003). Both fusions demonstrated the same pattern of expression over the different carbon sources tested suggesting that the regulatory effects on the native *sdhC* promoter were caused by the carbon source and were not a result of difference related to P_{530} and P_{247}.

The differences in β-galactosidase activity observed with the various carbon sources indicated that *sdhC* expression was under catabolic regulation. Strains grown with any of the three C_4-dicarboxylic acids (succinate, malate, and fumarate) evaluated showed similar expression levels to each other. The greatest levels of expression were observed with acetate and arabinose as sole carbon sources. Glutamate-grown cells had β-galactosidase activities lower than those grown in arabinose and acetate but higher than those grown with the C_4-dicarboxylic acids. The lowest levels of expression were observed for glucose- and pyruvate-grown cells (Figure 4.4).

To determine the influence of growth phase on expression, the strains carrying the *sdhC::lacZ* gene fusions were assayed over time using succinate (Figure 4.5) or glucose (Figure 4.6) as sole carbon sources. For cells grown in succinate, *sdhC::lacZ* expression seemed to decrease during mid log phase, only to increase during late-log phase. While the expression of the *sdh* promoter either

Figure 4.5. The effect of growth phase on the expression of the *sdh* promoter region of *S. meliloti* in succinate. The strains used in this study were: (□) RmG212, (■) Rm30187 (RmG212 *sdh44::lacZ*), and (■) Rm30188 (RmG212 *sdh375::lacZ*). Cells were grown in M9 supplemented with 15mM succinate and assayed for β-galactosidase activity at timed intervals. The bars represent β-galactosidase acitivity in Miller units (MU), values are the means of triplicate assays with standard errors indicated above the bars. OD_{600} readings are represented by closed circles.

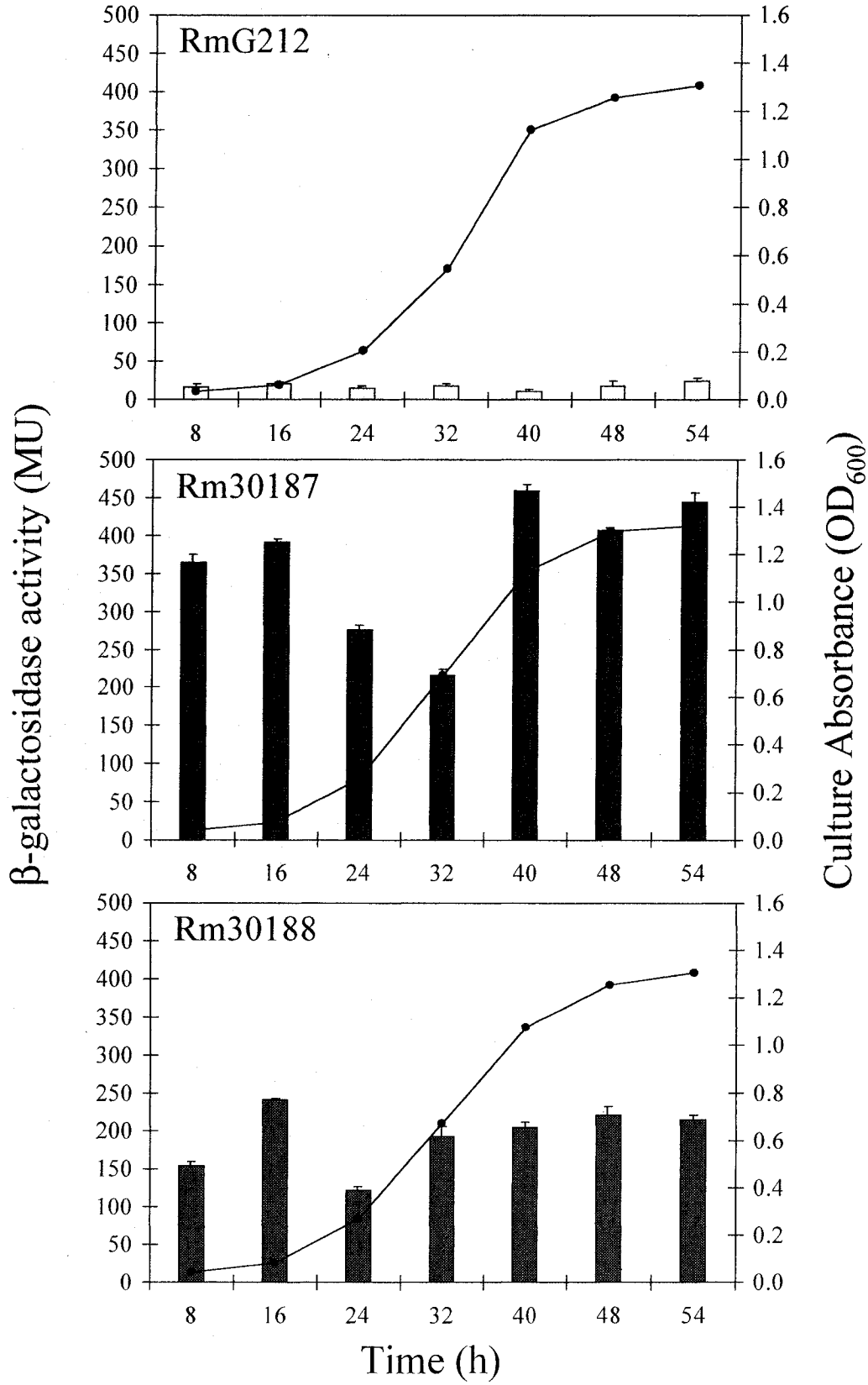
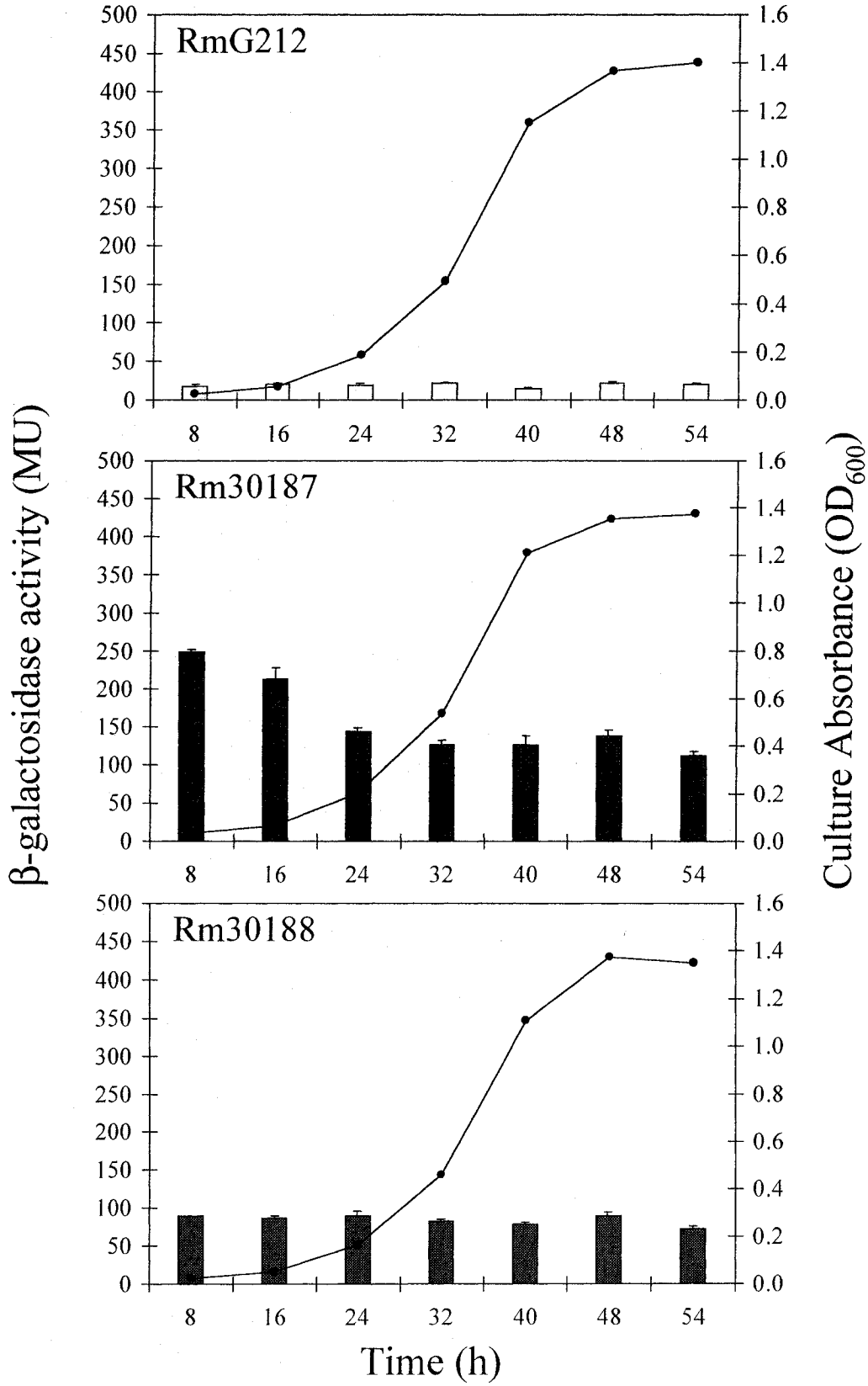

RmG212
Rm30187
Rm30188
β-galactosidase activity (MU)
Culture Absorbance (OD_{600})
Time (h)
0
50
100
150
200
250
300
350
400
450
500
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
8
16
24
32
40
48
54

Figure 4.6. The effect of growth phase on the expression of the *sdh* promoter region of *S. meliloti* in glucose. The strains used in this study were: (□) RmG212, (■) Rm30187 (RmG212 *sdh44::lacZ*), and (■) Rm30188 (RmG212 *sdh375::lacZ*). Cells were grown in M9 supplemented with 15mM glucose and assayed for β-galactosidase acitivity at timed intervals. The bars represent β-galactosidase acitivity in Miller units (MU), values are the means of triplicate assays with standard errors indicated above the bars. OD_{600} readings are represented by closed circles.


RmG212
Rm30187
Rm30188
β-galactosidase activity (MU)
Culture Absorbance (OD_{600})
Time (h)
0
50
100
150
200
250
300
350
400
450
500
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
8
16
24
32
40
48
54

remained constant (Rm30188) or showed a slight decrease (Rm30187) with increasing growth when assayed on glucose. These results demonstrated that *sdhC* expression was not growth dependent. The absence of β-galactosidase activity detected with RmG212 with both carbon sources and at all time intervals tested is an indication the activity observed was due to the transcriptional fusions.

DISCUSSION

The TCA cycle is believed to play an important role in symbiotic N_2-fixation as evident by the symbiotic phenotype generated by strains deficient for enzymes of the TCA cycle (Duncan and Fraenkel, 1979; McDermott and Kahn, 1992; Kahn *et al.*, 1995; Walshaw *et al.*, 1997; Dymov *et al.*, 2004). While most of the TCA enzymes have been extensively studied enzymatically, our knowledge of the regulation of the genes encoding these enzymes remains poor. The purpose of this study was to investigate the genetic organization and regulation of the *sdh* operon in *S. meliloti.*

The *S. meliloti* genome sequence revealed that the four subunits that make up SDH are encoded by four *sdh* genes ordered *sdhCDAB* which is closely followed by a putative *rho*-independent transcriptional stop sequence strongly suggesting the end of the operon at that position. RT-PCR analysis performed on the *sdh* gene cluster revealed that all four genes were cotranscribed, and 5'-RACE indicated the presence of a single promoter upstream of *sdhC*. A similar *sdh* operon organization was reported in *B. japonicum* (Westenberg and Guerinot, 1999). This order would

ensure that the anchor proteins synthesized first would be able to assemble and intergrate into the membrane providing an attachment point for the subsequently synthesized catabolytic subunits. It is interesting to note that a different operon organization has been reported for *E.coli* in which the *sdh* operon includes genes encoding for OGD subunits (*sucAB*) and SCS subunits (*sucCD*) (Cunningham and Guest, 1998). The fact that *E. coli sdh* appears to be regulated differently than in rhizobia could account for this difference in the organization of the *sdh* operon.

The transcriptional start site of the *sdhCDAB* gene cluster was determined by 5'-RACE analysis to begin 84 bp before the predicted *sdhC* translational start codon. The predicted -35 and -10 promoter region was found to share some homology with the only other rhizobial *sdh* promoter characterized to date, that of *B. japonicum* (Westenberg and Guerinot, 1999). The nucleotide sequence of the *sdh* promoter was also found to share some homology with sequences of other *S. meliloti* promoters as well as with the *sdh* promoters predicted for *E. coli* (Wilde and Guest, 1986) and *B. subtilis* (Melin *et al.*, 1987). To date, no consensus sequence has been found for *S. meliloti* promoters, the most conserved sequences appear to be located within the -35 promoter region, whereas very little homology is found in the -10 promoter region.

Having determined the transcriptional start site and the putative -35 and -10 regions of the promoter, transcriptional fusions were constructed to determine the impact of carbon source and growth phase on *sdh* expression. Two different transcriptional fusions were constructed, differing in the promoter sequence and distance between the *sdh* promoter and the *lacZ* gene. The inability to isolate any

chromosomal *sdh* transcriptional fusions that did not include the *sdh* promoter demonstrated that the promoter is required for cell viability. Because the fusions are generated by single recombination events, exclusion of the promoter region would lead to the formation of chromosomal transcriptional fusions that also generate lethal polar mutations effecting expression of *sdhA* and *sdhB*. This observation would help support our hypothesis that SdhA and SdhB are required for growth, discussed in greater detail in chapter 5.

Both chromosomal transcriptional fusions demonstrated the same expression patterns on the different carbon sources tested in both late log phase and time course experiments. However *sdh*375::*lacZ* showed consistently lower expression (Figure 4.4, 4.5 and 4.6). The same observations were found with *mdh* transcriptional fusions (Steven, 2003) and has been postulated to be related to an increased probability of premature transcriptional termination.

The significant differences in *sdh* promoter expression that were observed under different carbon sources suggest that the *sdh* operon is under metabolic regulation (Figure 4.4). The *sdh* promoter was shown to be stimulated by TCA cycle intermediates. The induction appears to increase with intermediates found in the upper branch of the TCA cycle (CIS to OGD). Acetate, arabinose and to a lesser extent glutamate were shown to induce *sdh* expression at a higher rate then the three C_4-carboxylic acids evaluated, while cells grown in glucose or pyruvate recorded the lowest levels of *sdh* expression. This expression pattern of high (acetate, arabinose, glutamate), medium (succinate, malate, fumarate) and low (glucose, pyruvate) were also reported for *mdh*, *sucC* and *sucD* transcriptional fusions in both

R. leguminosarum (Poole *et al.*, 1999) and *S. meliloti* (Steven, 2003).

Recently, the expression of *mdh* in *S. meliloti* was found to be growth phase regulated, which prompted us to evaluate if such a regulation mechanism was also found with *sdh*. Contrary to *mdh*, which demonstrated a significant increase in expression at the induction of log-phase growth, *sdh* expression demonstrated no such growth phase regulation. Even though expression was seen to fluctuate slightly over cell growth, the results were not deemed significant enough to justify growth phase regulation. This demonstrates that although SDH and MDH are involved in the same metabolic pathway, their respective genes appear to be regulated differently.

Connecting text

The previous Chapter (4) the *S. meliloti* operon that encodes SDH was characterized and shown to be metabolically but not growth-phase regulated. To help support these findings and to gain a better understanding of the role and regulation of SDH in *S. meliloti*, genetically well-defined *sdh S. meliloti* mutants were isolated and characterized. The work performed in Chapter 5 was designed to gain a better understanding of the role and regulation of SDH in *S. meliloti*. The *sdhCDAB* genes were shown to be co-transcribed, the *sdhC* transcription start site was mapped, and *lacZ* gene fusion studies of the regulation of the *sdh* operon by carbon source were done. The experimental work, accompanying analysis, and writing of this chapter was conducted by myself. Dr. Brian Driscoll provided critical reading of the chapter and suggested ways in which to improve the work.

Chapter 5. Isolation and characterization of succinate dehydrogenase mutants of *Sinorhizobium meliloti.*

SUMMARY

Based upon our previous characterization of the *sdh* operon (Chapter 4) and the *S. meliloti* genome sequence, the partial and complete *sdh* genes were cloned and subjected to transposon mutagenesis. Three *sdh* mutants (two in *sdhC* and one in *sdhD* gene) were isolated through EZ::TN mutagenesis followed by recombination into the *S. meliloti* genome, and to the best of our knowledge this is the first report of genetically well-defined *sdh* mutants within the rhizobia. The characterization of the mutants presented in this investigation supported our hypothesis that the *sdh* operon is under metabolic regulation. The *sdh* mutants also helped demonstrate that the total lack of SDH activity would be lethal to *S. meliloti* cells. The symbiotic phenotypes of the mutants indicated that a fully functional TCA cycle is required in N_2-fixing bacteroids.

INTRODUCTION

The TCA cycle plays a critical role in N_2-fixing bacteroids as demonstrated by the symbiotic phenotypes of reported mutants defective in TCA cycle enzymes which retain the ability to form nodules but are unable to fix N_2 (Johnson *et al.*, 1966; Finan *et al.*, 1981; McDermott and Kahn, 1992; Kahn *et al.*, 1995; Walshaw

et al., 1997; Dymov *et al.*, 2004). It has also been demonstrated that C_4-dicarboxylic acids, particularly succinate and malate are the carbon source supplied to bacteroids to support N_2-fixation (Streeter, 1991; Dunn 1998; Poole and Allaway, 2000). Rhizobial mutant strains with defective C_4-dicarboxylic acid transport (*dct*) systems have all been demonstrated to be unable to form effective N_2-fixing root nodules (Ronson *et al.*, 1981; Finan *et al.*, 1983; Yarosh *et al.*, 1989). The majority of the knowledge on the involvement of the TCA cycle in symbiotic N_2-fixation comes from enzymatic studies, hence, there is a great need for genetic studies involving well-characterized mutants.

The limited information that is known about the SDH enzyme complex and regulation of the genes that encode it in rhizobia, comes from studies conducted using undefined SDH mutants generated through NTG mutagenesis (Gardiol *et al.*, 1982; Gardiol *et al.*, 1987; Finan *et al.*, 1988). In the previous chapter (Chapter 4), the *S. meliloti* operon that encodes SDH was characterized and shown to be metabolically but not growth-phase regulated. To help support these findings and to gain a better understanding of the role and regulation of SDH in *S. meliloti*, genetically well-defined *sdh S. meliloti* mutants were isolated and characterized.

RESULTS

Isolation of Tn*5* insertions in the *sdh* genes

We attempted to isolate *sdh::*Tn*5* mutants via recombination of the plasmid-borne insertions into the *S. meliloti* genome using plasmid incompatibility. We had

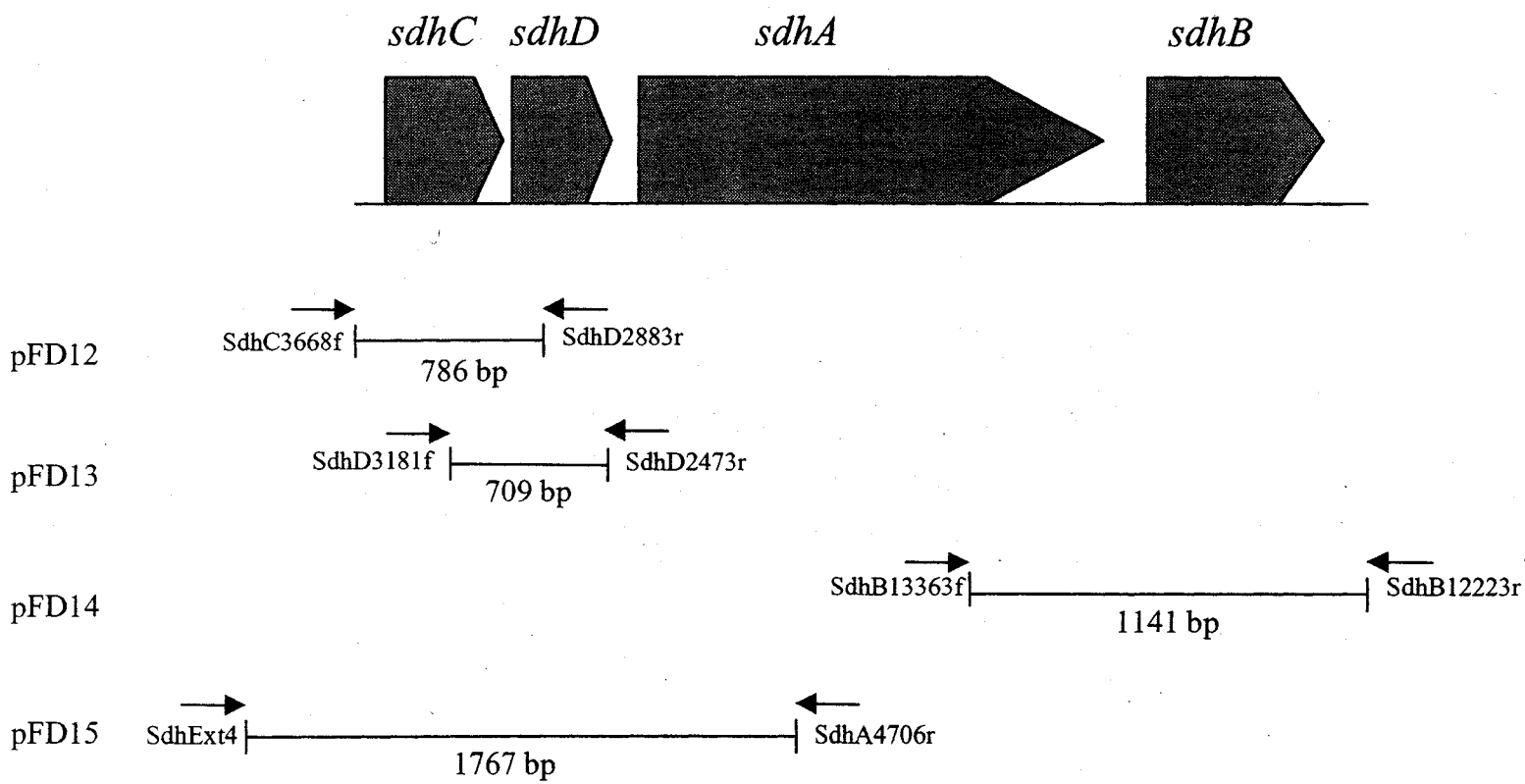
hypothesized that this might be impossible due to the high probability that *sdh::*Tn*5* insertions would generate absolute defective Sdh$^-$ strains, and that total absence of SDH would render such strains to be non-viable. Indeed, this appeared to be the case as no recombinant strains with Tn*5* insertions in any of the *sdh* genes could be isolated, and had not been reported in any previous study in which Tn*5* mutagenesis targeting genes of succinate metabolism had been done.

Based upon our previous characterization of the operon (Chapter 4) and the *S. meliloti* genome sequence (Garlibert *et al.*, 2000; Galibert *et al.*, 2001), primers were designed to amplify the partial and complete *sdh* genes. Primers sdhC3668f and sdhD2883r were used to amplify a 786 bp fragment containing the *sdhC* gene. Primers sdhD3181f and sdhD2473r were used to amplify a 709 bp fragment containing the *sdhD* gene. Primers sdhD5306f and sdhB2761r were used to amplify a 2562 bp fragment containing part of the *sdhA* gene. Finally, primers sdhB13363f and sdhB12223r were used to amplify a 1141 bp fragment containing the *sdhB* gene (Figure 5.1).

These PCR products, with the exception of the *sdhA* fragment which demonstrated poor PCR yields, were cloned into pGEM-T Easy resulting in plasmids pFD09 (*sdhC*), pFD10 (*sdhD*) and pFD11 (*sdhB*) respectively. The *sdh* fragments were then further sub-cloned into the *Eco*R1 site of pRK7813 generating plasmids pFD12 (*sdhC*), pFD13 (*sdhD*) and pFD14 (*sdhB*) (Figure 5.1). Tn*5* mutagenesis was carried out on all three plasmids and five *sdh*::Tn*5* insertions per plasmid were isolated.

The positions of each the Tn*5* insertions in the *sdh* genes were confirmed by

Figure 5.1. Cloning strategy for the *sdh* genes.


sdhC
sdhD
sdhA
sdhB
pFD12
SdhC3668f
SdhD2883r
786 bp
pFD13
SdhD3181f
SdhD2473r
709 bp
pFD14
SdhB13363f
SdhB12223r
1141 bp
pFD15
SdhExt4
SdhA4706r
1767 bp

Southern blot. Plasmid DNA was digested with *Eco*R1 and probed with the appropriate DIG-labeled probe (DIG*sdhC*, DIG*sdhD* or DIG*sdhB*) designed to complement the gene carried on the plasmid. An example of a typical Southern blot, in which mutagenized pFD12 probed with DIGSdhC is depicted (Figure 5.2). After confirming the insertion within the various *sdh* genes, the insertion sites were mapped (round-headed arrows in Figure 5.3) by DNA sequencing using the same primers used to generate the *sdh* gene fragments.

Isolation of EZ::TN *sdh* mutants

Tn*5* mutagenesis of the plasmids yielded only insertions that rendered recombinant strains non-viable, therefore the isolation of *sdh* mutants required the use of a system that yielded insertions that did not cause polar effects in the *sdh* operon. The EZ::TN^{TM} <Kan-2> (EZ::TN) system has a 1.2-kb DNA element derived from Tn*903* which carries a Km^{r}Nm^r cassette but no transposase gene. Since the 3'-end of the Km ORF has no "overtly visible" transcription terminators, there can be some run-through or "leaky" transcription 3' from the transposon.

First, PCR was used to amplify fragments of the *sdh* operon that were subsequently cloned into a pGEM-T Easy vector. Primers sdhExt4 and sdhA4706r were used to amplify a 1767 bp fragment that contained the promoter region, *sdhC*, *sdhD* and *sdhA* (713 bp upstream of the *sdhC* ATG start codon to 262 bp within *sdhA*). This fragment was cloned into pGEM-T Easy to generate plasmid pFD15. Plasmids pFD12, pFD13, pFD14 and pFD15 were subjected to EZ::TN mutagenesis. The restriction endonuclease digestion patterns of the plasmids were

Figure 5.2. Southern hybridization of putative *sdhC* Tn*5* transposon mutants. Plasmid DNA was restricted with *EcoR*1 and probed with DIGSdhC. Lane 1, pFD12; lane 2, pFD17; lane 3, pFD18; lane 4, pFD19; lane 5, pFD149, lane 6, pFD20. The predicted size for the EcoR1 fragment with and without the Tn5 insert are indicated by arrows.

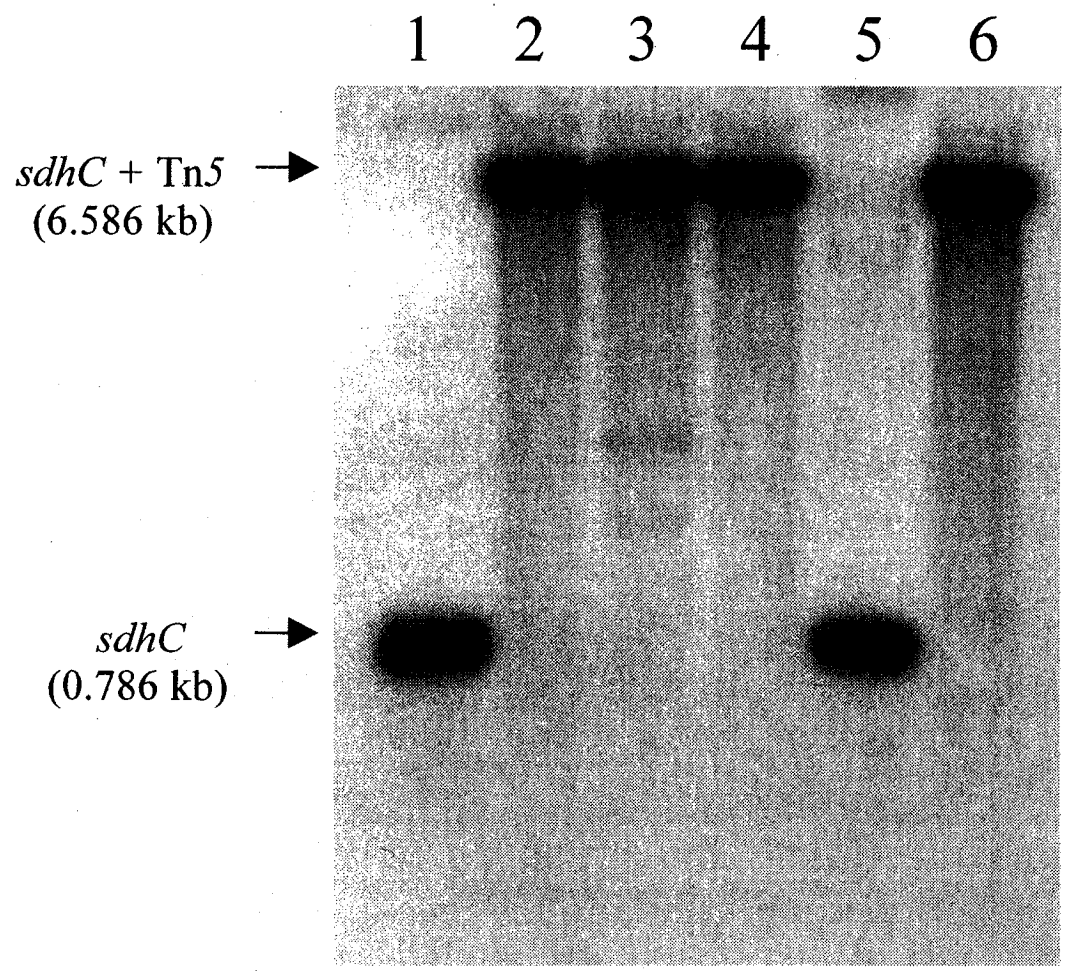
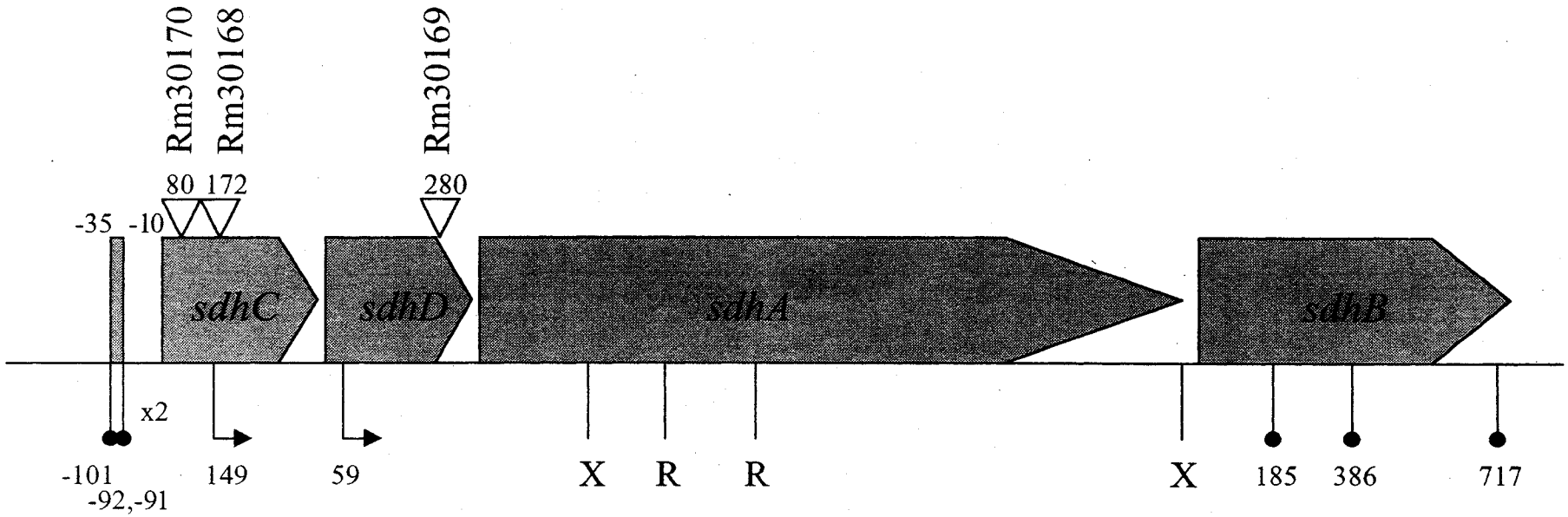

1 2 3 4 5 6
sdhC + Tn5
(6.586 kb)
sdhC
(0.786 kb)

Figure 5.3. Genetic map of the *sdh* operon and transposon insertion sites. Genes are represented by open arrow boxes with the orientations of the arrows representing the direction of transcription. The -10 and -35 putative promoter region is designated by a labeled box. Transposon insertion sites of sequenced mutants are indicated by arrows, round headed Tn*5*, and solid headed triangles for Tn*5*-B20. The EZ::TN transposomes insertion sites are represented by open triangles. The relevant restriction sites shown are *Eco*R1 (R), *Xho*1 (X). The lower closed box represents the DIG- labeled probe used for Southern hybridization.



DIG *sdhC* probe

1 kb

used to verify the position of the insertions, and only plasmids with insertions within the *sdh* fragments were retained. A total of 28 mutated plasmids (pFD23 to pFD50) were generated with EZ::TN transposome inserts: 2 in pFD12 (*sdhC*), 5 in pFD13 (*sdhD*), 5 in pFD14 (*sdhB*), and 16 in pFD15 (*sdhC, sdhD,* part of *sdhA*).

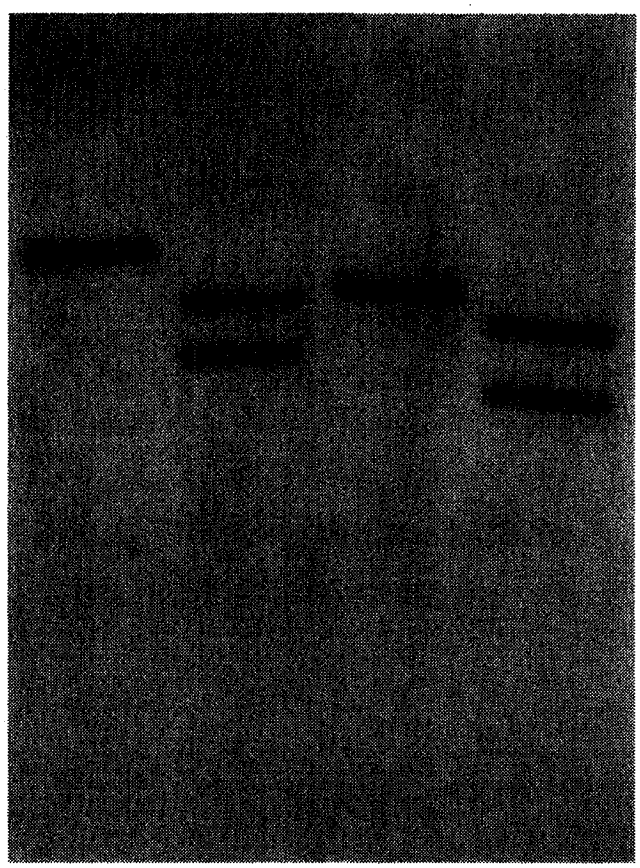
Six plasmids (pFD23 to pFD28) carrying EZ::TN insertions in pFD15 were used to generate *S. meliloti sdh* mutants by recombination. The fragments were subcloned into the *Not*1 restriction site of pJQ200sk resulting in plasmids pFD51 to pFD56. The pJQ200sk vector carries the *sacB* gene, encoding levansucrase which is lethal in Gram-negative bacteria. After transferring each of the plasmids into Rm1021 by conjugation, strains resulting from double homologous recombination events between the plasmids and the chromosome were selected on media containing Nm and 5% sucrose (Quandt and Hynes, 1993). The homogenotes were then screened for growth on minimal media containing glucose and succinate as sole carbon sources, respectively. Using this methodology, three putative *sdh* mutants were isolated: Rm30168 (*sdhC*), Rm30169 (*sdhD*) and Rm30170 (*sdhC*). Each of these strains was able to grow on M9 glucose but not on M9 succinate (Suc$^-$).

Mapping of the putative *sdh* mutants

A Southern blot was performed to confirm that the isolated strains carried EZ::TN insertions and to locate the insertions within the *sdh* operon. Genomic DNA was isolated, restricted and probed using a DIG-labeled *sdhC* probe (Figure 5.4) generated using primers sdhC3668f and sdhD2883r, as described in Chapter 2. Probing of a Southern blot of *Eco*R1-digested genomic DNA demonstrated a band

Figure 5.4. Southern hybridization of putative *sdh* mutants. Genomic DNA was restricted with *Xho*1 and probed with DIGSdhC probe. Lane 1, Rm1021; lane 2, Rm30168; lane 3, Rm30169; lane 4, Rm30170.

1 2 3 4

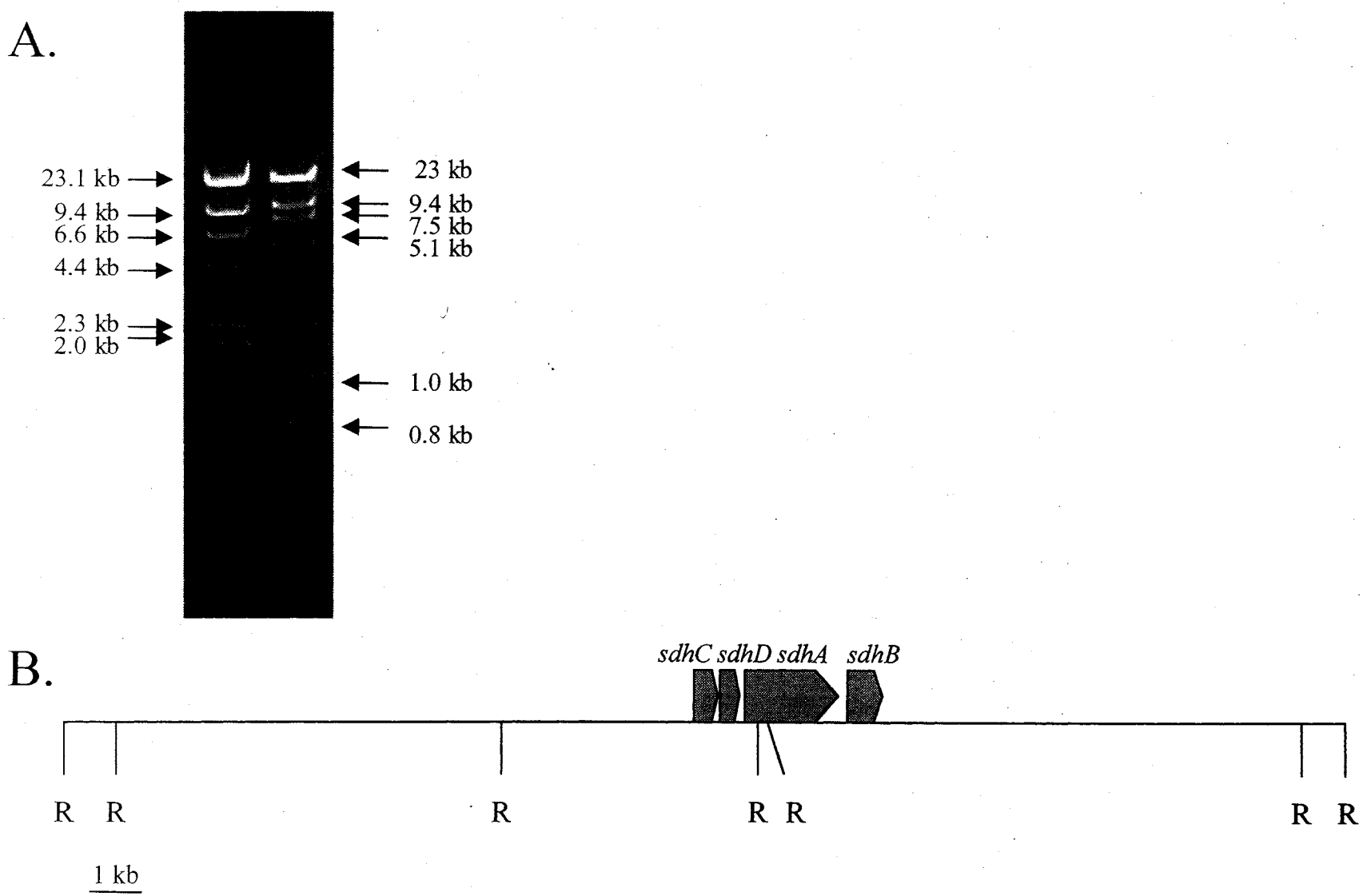


shift between the mutants and the wild-type indicative of a EZ:TN insertion (data not shown). A Southern blot of *Xho*1-digested genomic DNA was also done as the restriction endonuclease was predicted to be able to cut within the EZ::TN transposome but not within s*dhC*. As expected only one band, corresponding to *sdhC*, was observed with wild-type Rm1021 DNA (Figure 5.4). The presence of two bands in the lanes associated with Rm30170 and Rm30168 indicated that the EZ::TN insertion was within *sdhC*, whereas the presence of one band in Rm30169 suggested that the insertion was located outside of *sdhC*. The exact location of the EZ::TN insertions were determined by DNA sequencing (Figure 5.3).

Isolation and characterization of the complementing cosmid pFD71

Heterologous complementation of the three *sdh* mutants isolated was used to isolate the wild-type *S. meliloti sdh* operon. Complementation of the *sdh* mutants with a *S. meliloti* cosmid (pLAFR1) library (Friedman *et al.*, 1982) was performed as described in Chapter 2. Strains Rm30168, Rm30169 and Rm30170 were unable grow on M9 succinate plates. Several complementing cosmids were isolated that restored the wild-type phenotype in the *sdh* mutants when grown on M9 succinate. All cosmid clones isolated had the same restriction endonuclease patterns and therefore a representative complementing cosmid clone was retained for further analysis and designated pFD71. The *Eco*R1 restriction pattern and genetic map of pFD71 is depicted in Figure 5.5. DNA sequencing revealed that pFD71 carried a 24,146 nt fragment of the *S. meliloti* genome spanning nt 3,322,470 to nt 3,346,614 of the *S. meliloti* chromosome, which includes the entire *sdh* operon (Figure 5.5).

Figure 5.5. Physical and genetic map of the complementing cosmid pFD71. **A.** Restriction endonuclease analysis of pFD71. Plasmid DNA was restricted with *Eco*R1 and subjected to agarose gel electrophoresis. Lane 1, λ *Hind*III standard molecular weight; lane 2, pFD71. **B.** Genetic map of pFD71. *Eco*R1 restriction sites are indicated by (R).


A.
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
23 kb
9.4 kb
7.5 kb
5.1 kb
1.0 kb
0.8 kb
B.
sdhC sdhD sdhA sdhB
R R R R R R R
1 kb

The cosmid was introduced by conjugation into the three *sdh* mutants resulting in strains Rm30181, Rm30183, and Rm30186 respectively.

Tn*5*-B20 mutagenesis of pFD71

The complementing plasmid pFD71 was subjected to random Tn*5*-B20 in the hopes of generating plasmid borne *sdh* transcriptional fusions that could then be used in regulation studies. Plasmids with insertions within the *S. meliloti* insert were chosen based on restriction endonuclease pattern analysis. A total of 72 plasmids have been isolated to date designated pFD77 to pFD148. The insertion locations as well as the orientation of the *lacZ* gene were mapped by DNA sequencing using primers (ptn5, placZ) designed to amplify outwards from the Tn*5*-B20 ends. Two plasmids (pFD123, pFD127) were found to have Tn*5*-B20 insertions within the *sdh* operon in the proper orientation (see Figure 5.3). Insertions in plasmids pFD123 and pFD127 mapped to *sdhC*149 and *sdhD*59 respectively. Although the plasmids have not been utilized as of yet, they represent genetic tools that can be very useful in future experiments. In addition, as with the previously-isolated *sdh*::Tn*5* insertions, these insertions could not be homogenotized into the *S. meliloti* chromosome further supporting our previously mentioned hypothesis of polar mutation effects on *sdhA* and *sdhB* resulted in non-viable strains.

Growth phenotype of *sdh* mutants and complemented strains.

The growth phenotypes of the *sdh* mutants were tested by plating the strains

on M9 minimal media with different carbon sources (Table 5.1, Figure 5.6). Rm1021 was able to utilize all of the carbon sources evaluated. Strains Rm30168, Rm30169, and Rm30170 showed similar growth patterns on the different carbon sources. As expected none of the mutant strains were able to grow on M9 succinate. They were also unable to utilize acetate, arabinose or pyruvate as sole carbon source. The mutants were able to grow with glucose, malate and fumarate as sole carbon source, however the growth was much slower than observed with the wild-type Rm1021, taking up to 7-10 days for visible colonies to appear. The complementing cosmid pFD71 was able to restore to wild-type levels the growth phenotype of all three mutants (strains Rm30181, Rm30183, Rm30186).

Growth curves were performed in liquid minimal media to further characterize the *sdh* mutants. Cultures were grown in M9 media supplemented with 15 mM carbon source. No significant differences could be seen between the *sdh* mutant strains. The growth curves further supported the plate growth phenotype observations in that the *sdh* mutants were unable to use pyruvate (Figure 5.9), acetate (Figure 5.10), arabinose (Figure 5.11), glutamate (Figure 5.12), or succinate (Figure 5.13) as sole carbon sources. The carbon sources that were able to be utilized by the *sdh* mutants included: glucose (Figure 5.8) and C_4-dicarboxylic acids fumarate (Figure 5.14) and malate (Figure 5.15). Even when growth was observed, the growth was significantly slower than observed with the wild-type.

The mutants seem to show the best growth on malate, followed by glucose and finally fumarate. The mutants also demonstrated a much slower growth rate compared to the wild-type Rm1021 when grown in complex medium (Figure 5.7).

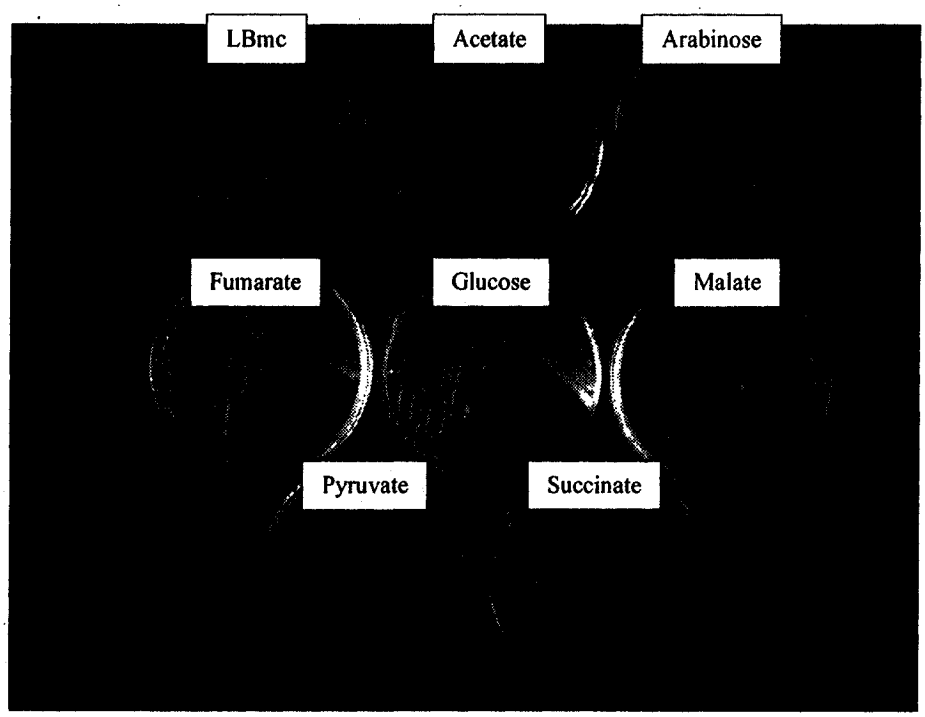
Table 5.1. Growth phenotype of wild type and *sdh* mutant *S. meliloti* strains on different carbon sources.

	Growth phenotype on carbon source[a]								
Strain	LB	Succinate	Acetate	Arabinose	Glutamate	Glucose	Pyruvate	Malate	Fumarate
Rm1021	++	++	++	++	++	++	++	++	++
Rm30168	+	-	-	-	-	+	-	+	+
Rm30169	+	-	-	-	-	+	-	+	+
Rm30170	+	-	-	-	-	+	-	+	+
Rm30181	++	++	++	++	++	++	++	++	++
Rm30183	++	++	++	++	++	++	++	++	++
Rm30186	++	++	++	++	++	++	++	++	++

[a] Strains were grown on M9 plates supplemented with 15 mM of the different carbon sources evaluated. A positive growth phenotype corresponds to the appearance of colonies within 14 days of plating.

Figure 5.6. Growth phenotypes of Rm1021 and *sdh* mutants on several carbon sources. **A.** Photograph of growth phenotype of Rm1021 on M9 plates supplemented with 15 mM carbon source. Carbon sources evaluated are indicated above the plates. **B.** Photograph of growth phenotype of Rm30168 on M9 plates supplemented with 15 mM carbon source. Carbon sources evaluated are indicated above the plates. Strains Rm30169 and Rm30170 are not shown but demonstrated growth similar to Rm30168, while complemented strains (Rm30181, Rm30183, Rm30186) were comparable to Rm1021.

A.


LBmc
Acetate
Arabinose
Fumarate
Glucose
Malate
Pyruvate
Succinate

B.

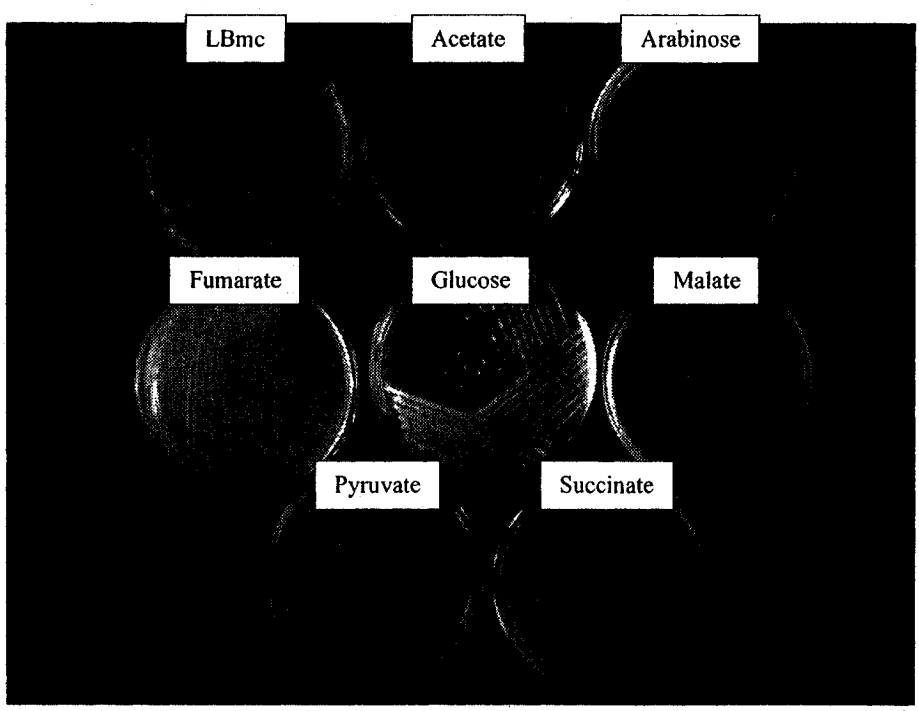

LBmc
Acetate
Arabinose
Fumarate
Glucose
Malate
Pyruvate
Succinate

Figure 5.7. Growth curve of *S. meliloti* wild type and mutant strains in complex LB media. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

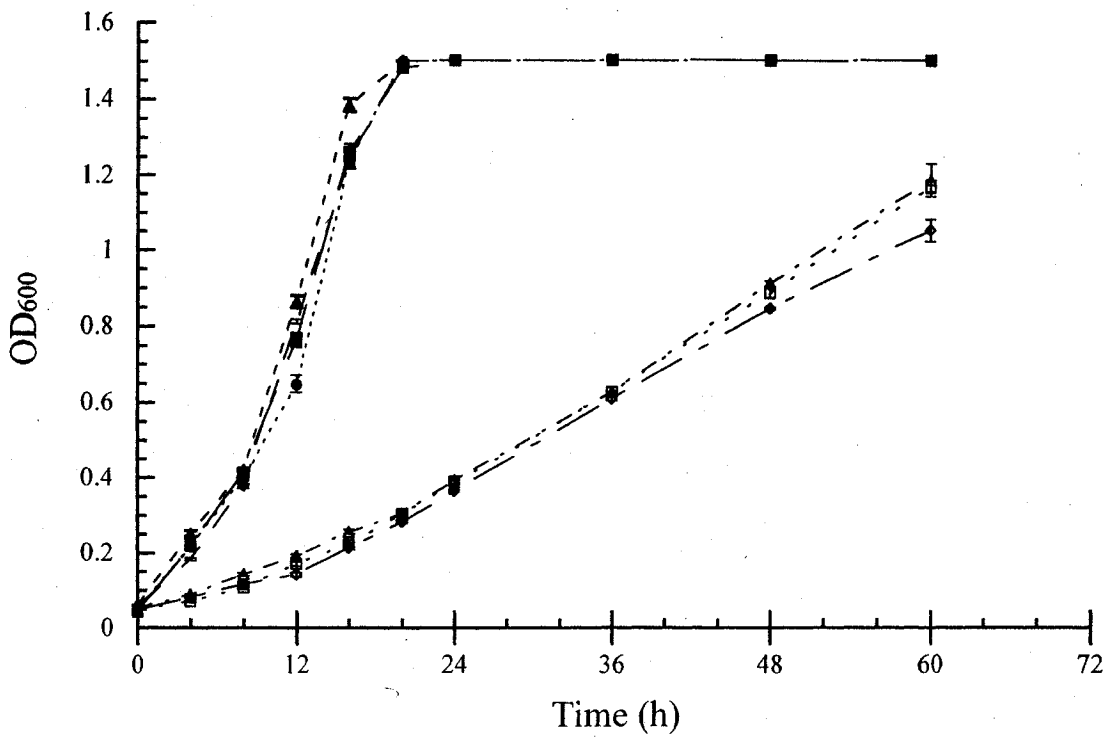

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.8. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with glucose as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

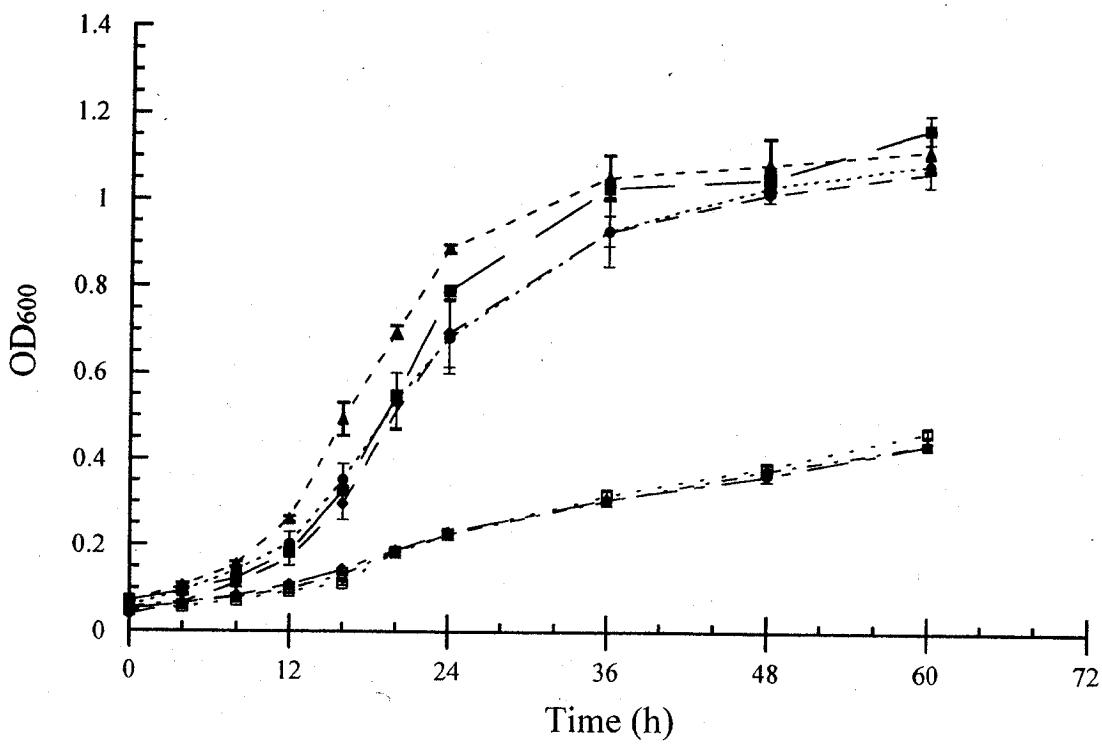

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.9. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with pyruvate as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

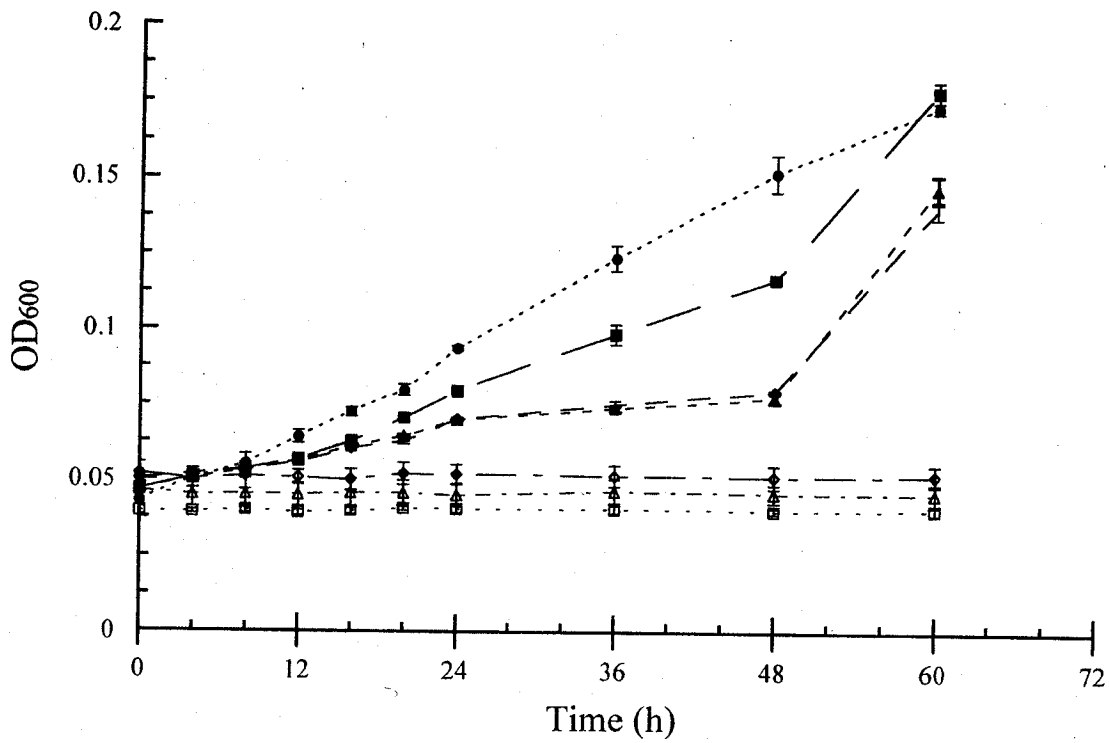

0.2
0.15
0.1
0.05
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.10. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with acetate as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

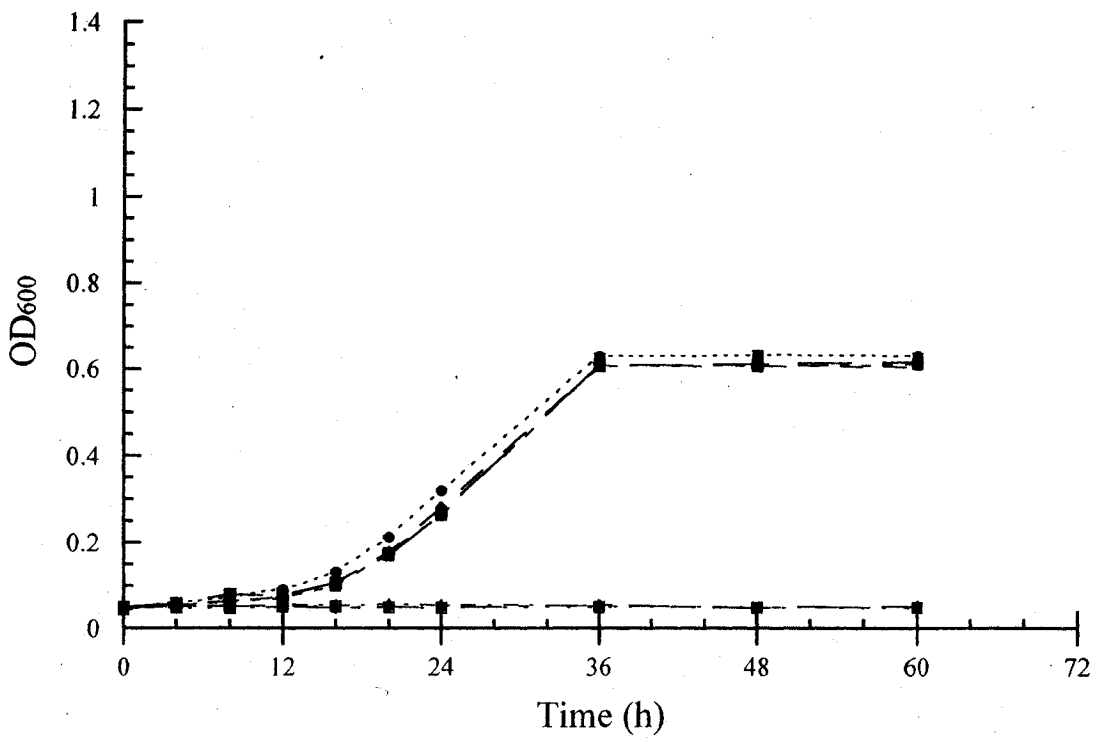

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.11. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with arabinose as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

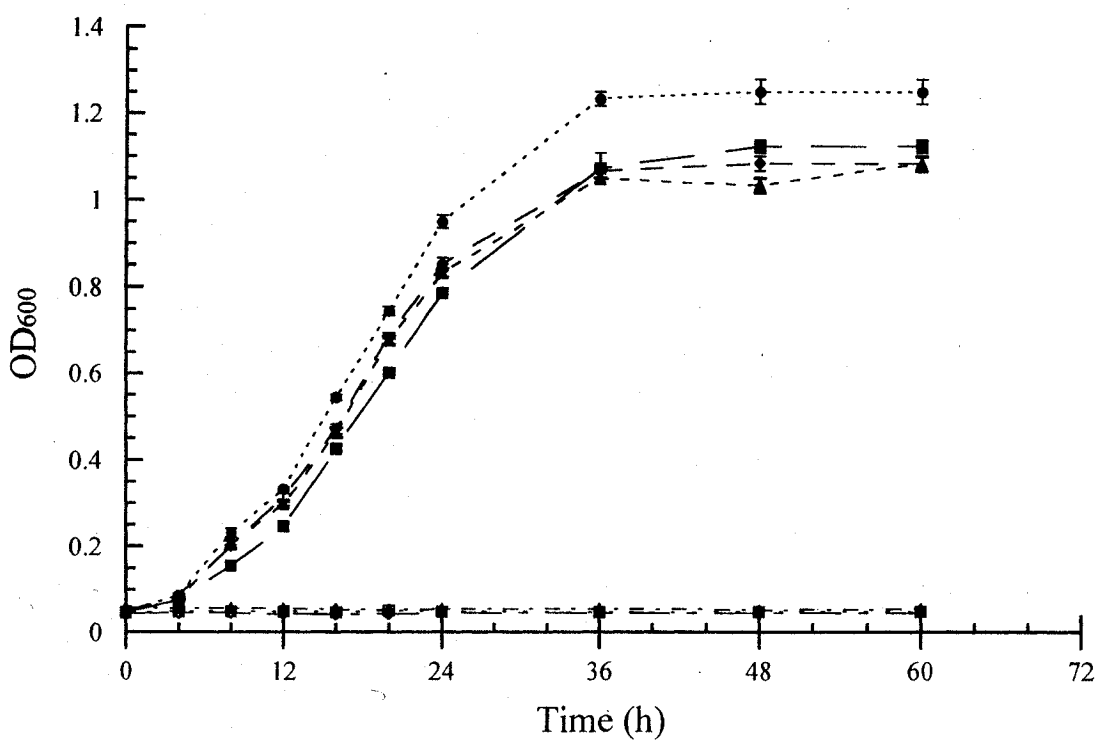

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.12. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with glutamate as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

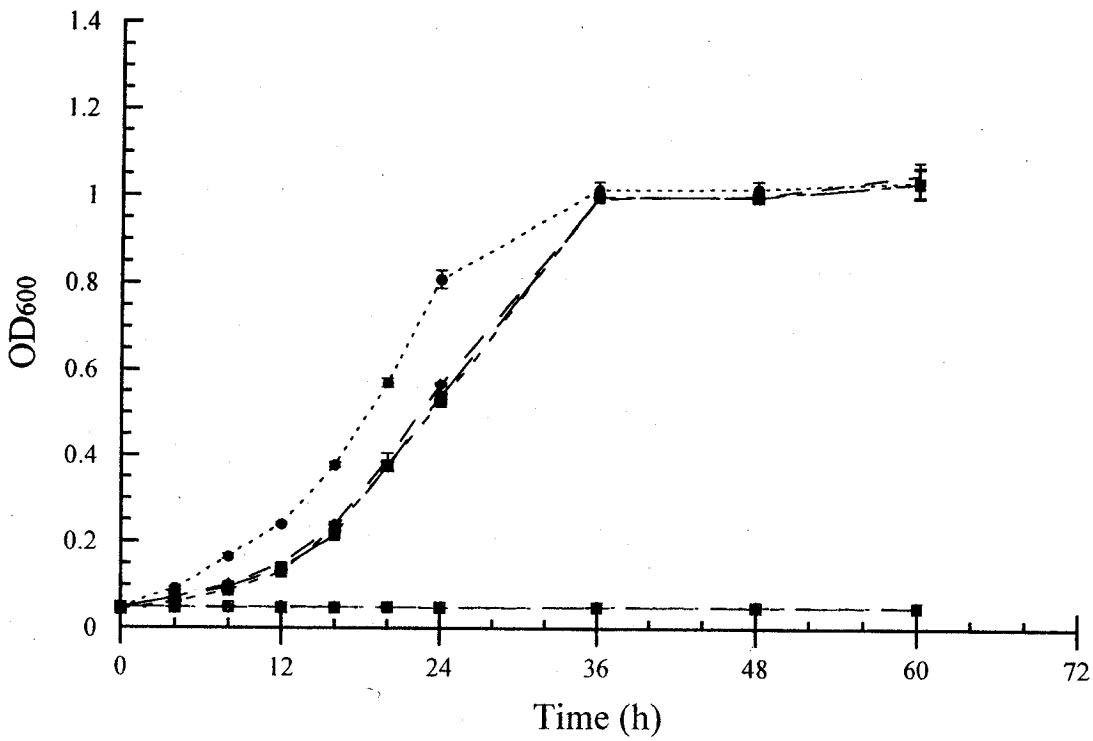

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.13. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with succinate as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

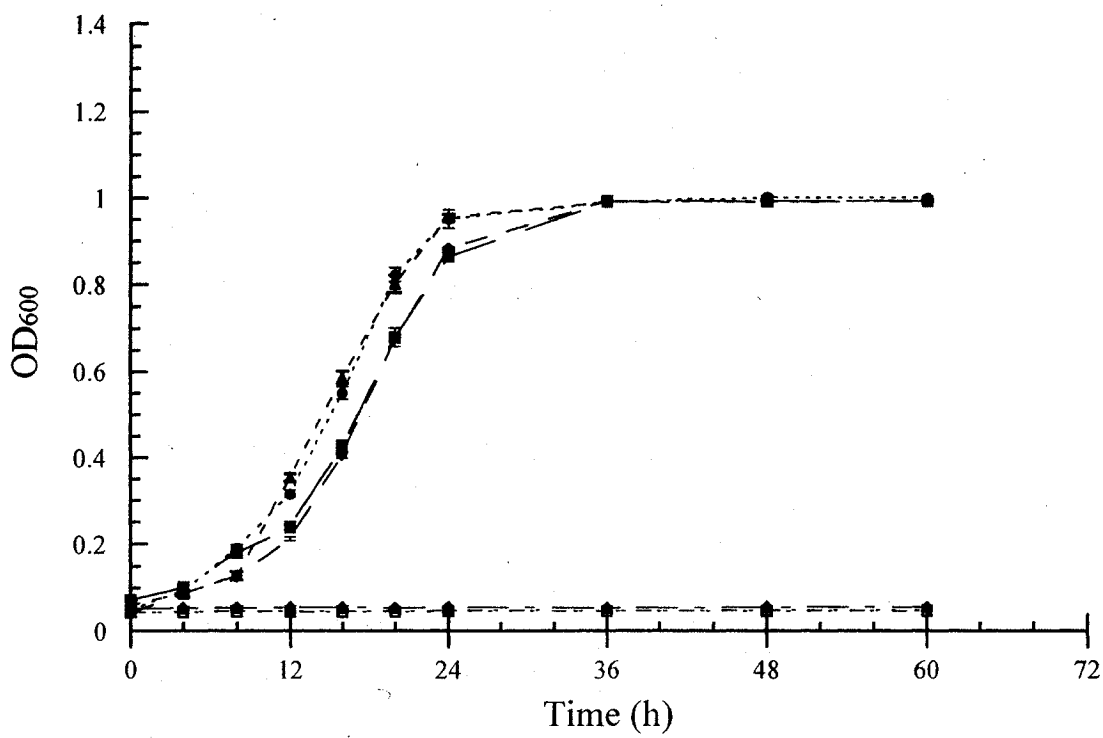

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.14. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with fumarate as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.

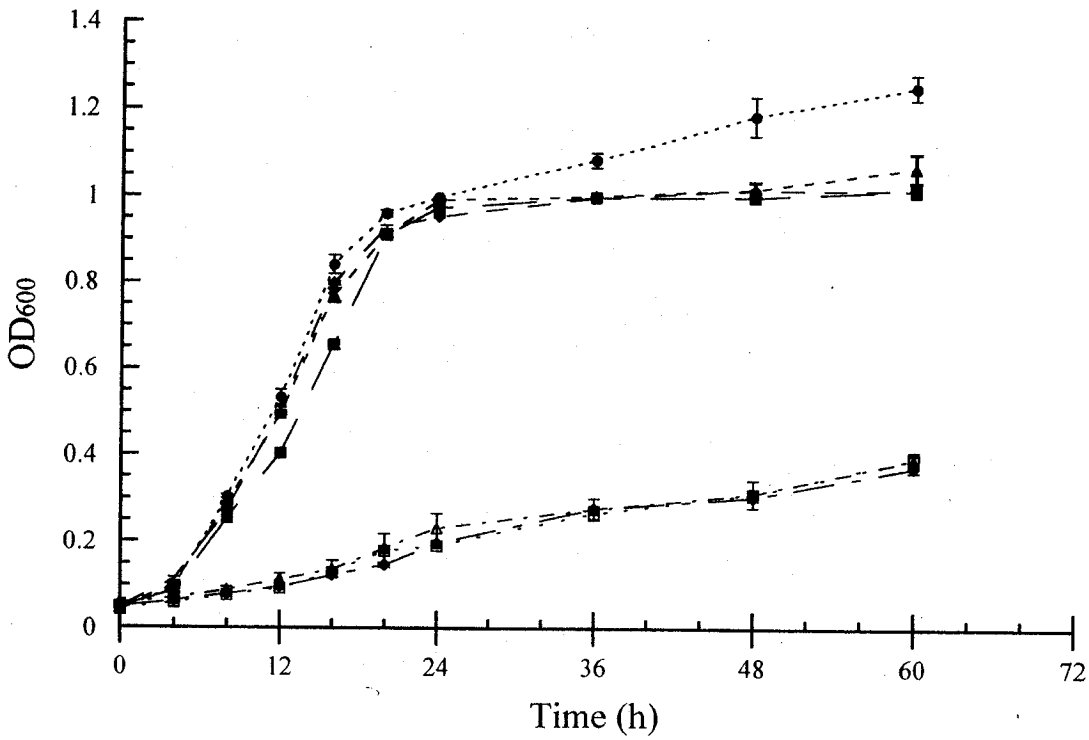
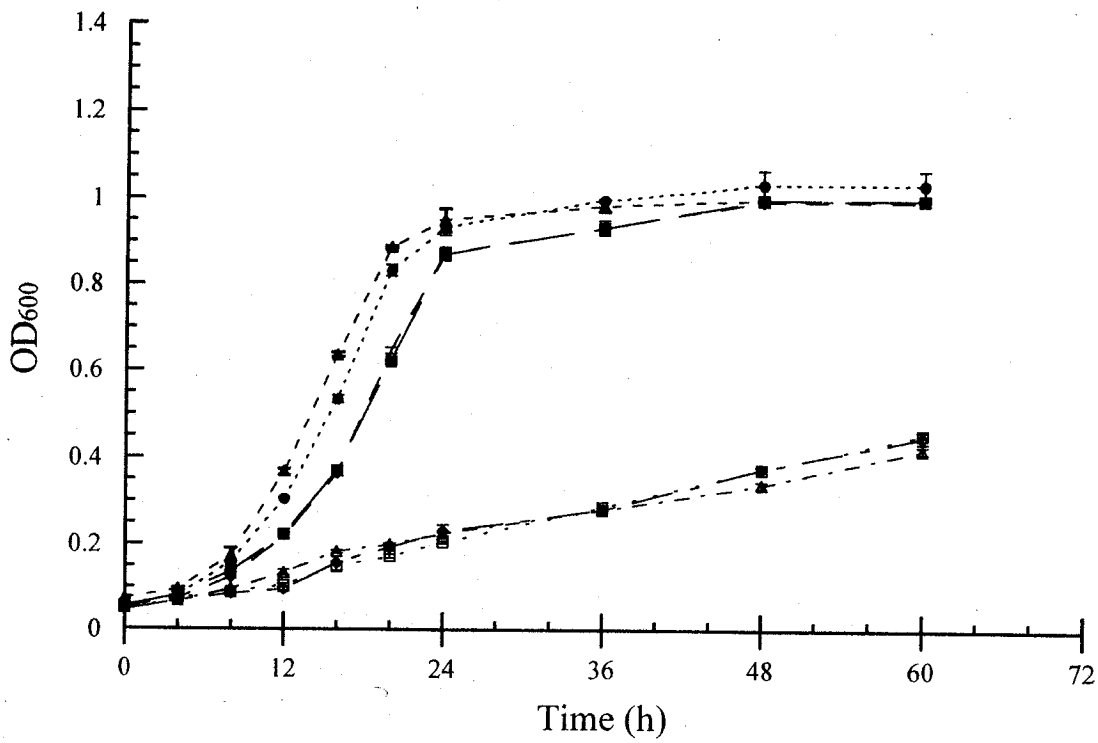

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

Figure 5.15. Growth curve of *S. meliloti* wild type and mutant strains in M9 media with malate as sole carbon source. Wild type Rm1021 (●), *sdh* mutant strains Rm30168 (□), Rm30169 (◊), Rm30170 (Δ) , *sdh* mutant strain complemented with pFD71 Rm30181 (■), Rm30183 (◆), Rm30186 (▲). Values represent means of triplicate assays, standard errors are indicated by vertical bars.


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
12
24
36
48
60
72
Time (h)

The growth assays also demonstrated the ability of cosmid pFD71, which carries a wild-type copy of the *sdh* operon, to complement the *sdh* mutants on all carbon sources evaluated as seen by the similar growth rates of strains Rm30181, Rm30183, and Rm30186 compared to Rm1021 (Figure 5.7 to 5.15).

Enzymatic characterization of *sdh* mutants and complemented strains

Initial enzymatic characterization of the mutants using crude cell extracts revealed that they retained some SDH activity (Table 5.2). Although SDH activity was reduced by more than half, it was not absent as expected. When the same SDH enzyme assay was performed using membrane fractions instead of crude cell extracts, no SDH activity was detected in all three mutants. A similar finding was reported from studies using an *E. coli sdh⁻ fdr⁻* double mutant carrying *sdhAB* on a plasmid (Nakaruma, *et al.*, 1996). Using this strain, it was shown that SDH activity could be detected in crude cell extracts but not in membrane preparations, demonstrating that only the two catabolic subunits, SdhA and SdhB, are needed to for SDH activity.

Enzyme assays also revealed that MDH activity in the *sdh* mutants was 10-fold higher compared to wild-type (Table 5.2). The mutant strains also showed elevated OGD, SCS and to a lesser extent ICD activities (Table 5.2). The SDH activity was restored to near wild-type levels in the complemented strains in both crude cell extracts and membrane fractions. The introduction of pFD71 also restored enzyme activities of MDH, OGD, ICD, SCS to levels comparable to Rm1021 (Figure 5.7 to 5.15)

Table 5.2. Enzyme activities of *S. meliloti* wild type and *sdh* mutant strains.

Strain	Enzyme specific activity[a]					
	SDH		MDH	IDH	OGD	SCS
	Crude	Membrane				
Rm1021	291 ± 5	111 ± 12	519 ± 2	87.4 ± 4.9	43.6 ± 0.8	54.9 ± 2.2
Rm30168	112 ± 7	0 ± 0	4829 ± 5	155 ± 8	122 ± 4	386 ± 10
Rm30169	113 ± 10	0 ± 0	4995 ± 5	125 ± 1	122 ± 5	354 ± 32
Rm30170	141 ± 8	0 ± 0	4582 ± 9	181 ± 2	120 ± 6	359 ± 38
Rm30181	232 ± 9	73 ± 7	391 ± 3	137 ± 2	36.0 ± 2.2	76.5 ± 6.0
Rm30183	214 ± 16	80 ± 6	421 ± 0	143 ± 4	28.6 ± 1.7	78.5 ± 8.2
Rm30186	219 ± 21	65 ± 8	382 ± 2	154 ± 9	21.5 ± 3.0	96.0 ± 10.5

[a] Specific activities (nmol per minute per mg protein) expressed as mean ± standard error of triplicate assays of each sample.

Transcription analysis using RT-PCR

The observation of SDH activity in crude cell extracts indicated that *sdhA* and *sdhB* were being expressed in both the *sdhC* and *sdhD* mutants. Therefore to confirm that the EZ::TN insertions did not cause polar mutations, we performed RT-PCR using RNA isolated from the *sdh* mutants. The presence of bands in the RT-PCR performed with primers designed to amplify the *sdhD-sdhA* intergenic region demonstrated the expression of *sdhAB* transcript in the *sdhC* and *sdhD* mutants (Figure 5.16). The band shift seen with Rm30169 was expected since the insertion site is located within the amplified *sdhD-sdhA* fragment and therefore resulted in a product 1221 bp (size of the EZ::TN transposome) larger than the predicted size of the *sdhD-sdhA* amplified fragment (618 bp). From the size of this band it is also apparent that the entire EZ::TN sequence is cotranscribed with the *sdh* operon in these strains.

Symbiotic phenotype

The symbiotic phenotypes of the *sdh* mutants were determined using alfalfa plants as described in Chapter 2. The shoot dry weights of plants inoculated with the *sdh* mutants Rm30168, Rm30169 and Rm30170 were significantly lower compared to plants inoculated with the wild-type Rm1021 (Table 5.3). The appearance of the plants, chlorotic and stunted, resembled the uninoculated control (Figure 5.17). Observation of the root systems revealed small white nodules (Figure 5.18) suggesting that all three mutants retained the ability to nodulate, however the nodules were unable to fix N_2. Because succinate is believed to be the main carbon

Figure 5.16. Transcription analysis of the *sdhCDAB* operon in *sdh* mutant strains using RT-PCR. Agarose gel electrophoresis of RT-PCR products that span the *sdhD-A* intergenic regions of the *sdhCDAB* operon. Lanes 1 and 8, 100 bp ladder; lane 2, no RNA; lane 3, no reverse transcriptase; lane 4, Rm1021; lane 5, Rm30168; lane 6, Rm30169; lane 7, Rm30170.

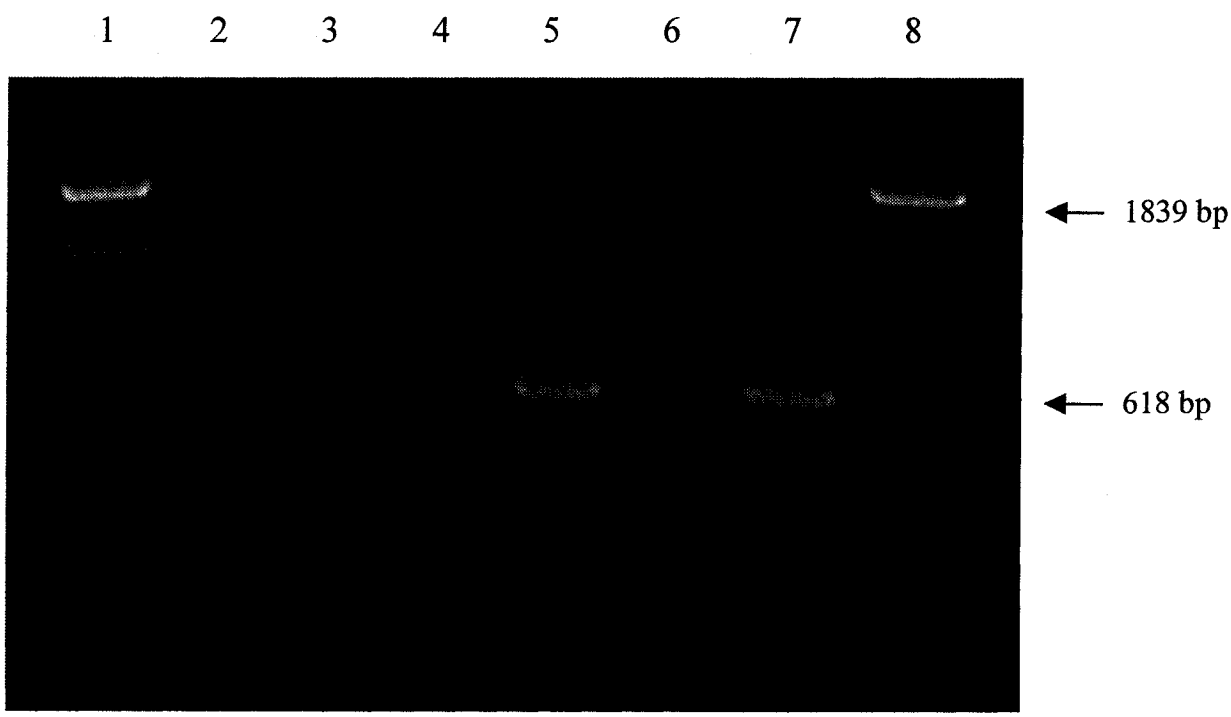

1
2
3
4
5
6
7
8
1839 bp
618 bp

Table 5.3. Shoot dry weight values of alfalfa plants inoculated with *Sinorhizobium meliloti* mutants.

Inoculated strain	SDW[a]	%Wild type	Symbiotic phenotype
Rm1021	65.1 ± 3.2	100	Fix^+
Uninoculated	6.69 ± 0.11	10	Fix^-
Rm30168	7.01 ± 0.09	11	Fix^-
Rm30169	7.93 ± 0.35	12	Fix^-
Rm30170	7.17 ± 0.61	11	Fix^-
Rm30181	37.5 ± 1.5	58	Fix^+
Rm30183	33.7± 2.6	52	Fix^+
Rm30186	35.7 ± 1.3	55	Fix+

[a]Shoot dry weight (SDW) values are presented as mean mg per plant ± standard error of the mean. Data are of one experiment and were consistent upon replication.
[b]The ability to fix nitrogen (Fix) was determined by evaluation of the SDW and appearance of plants. Fix^+ plants appeared green and healthy (similar to those inoculated with Rm1021), Fix^- plants appeared chlorotic and stunted (similar to plants not inoculated with *S. meliloti* (uninoculated controls).

Figure 5.17. Photograph of the symbiotic phenotype of wild type and *sdh* mutant *S. meliloti* strains. Alfalfa plants were inoculated with wild type (Rm1021), *sdh* mutants (Rm30168, Rm30169, Rm30170), *sdh* mutants complemented with pFD71 (Rm30181, Rm30183, Rm30186), or uninoculated (Control). Alfalfa plants were grown for 28 days post-inoculation.


Control
Rm30068
Rm30069
Rm30070
Rm1021


Control
Rm30081
Rm30083
Rm30086
Rm1021

Figure 5.18. Photograph of nodules isolated from alfalfa plants 28 days after inoculation with wild type or *sdh* mutant *S. meliloti* strains. **A.** Nodule isolated from an alfalfa plant inoculated with Rm1021. This nodule is representative of nodules isolated from plants inoculated with *sdh* mutant strains complemented with pFD71 (Rm30181, Rm30183, Rm30186). **B.** Nodule isolated from an alfalfa plant inoculated with Rm30168. This nodule is representative of nodules isolated from alfalfa plants inoculated with *sdh* mutant strains Rm30169 and Rm30170.


B

source supplied to the bacteroids, disruption of *sdh* would expectedly disrupt N_2fixation. The fact that the *sdh* mutants demonstrated a reduction of SDW of close to 90% compared to Rm1021 supports this hypothesis.

The complementing cosmid pFD71 was able to partly restore the wild-type phenotype as demonstrated by the increased SDW of strains Rm30181, Rm30183 and Rm30186 (Table 5.3). Plants inoculated with these complemented strains resulted in healthy looking green plants with well formed pink nodules not unlike those inoculated with Rm1021 (Figure 5.17 and 5.18). The plasmid was not able to restore the phenotype completely as evident by the lower SDW (Table 5.3). Previous studies have demonstrated the instability of laboratory plasmids in free-living and bacteroids due to the absence of antibiotic selection for plasmid maintenance (Weinstein *et al.*, 1992; Driscoll and Finan, 1997). In one study, of the colonies recovered from nodules of alfalfa plants inoculated with a strain carrying a pLARF1 complementing clone, only 13% retained the plasmid (Dymov *et al.* 2004). Therefore it is likely that the differences seen between the complement strains Rm30181, Rm30183, and Rm30186 could be attributed to loss of plasmid.

DISCUSSION

The isolation of *sdh* mutants has never been reported in previous studies using transposon mutagenesis (Finan *et al.*, 1981; Dymov *et al.*, 2004). One study screened over 4000 independent Tn-5 mutants able to grow on glucose but not succinate as sole carbon source, but none were *sdh* mutants. This group had been

able to isolate an *R. leguminosarum* SDH defective mutant, however, using NTG mutagenesis (Finan *et al.*, 1981). The only known *S. meliloti* SDH mutant was also isolated through NTG mutagenesis (Gardiol *et al.*, 1982). The mutation in this strain was presumed, but never demonstrated, to be within one of the *sdh* genes because the strain lacked SDH enzyme activity and was unable to grow on succinate.

We hypothesized that, as the above NTG-derived mutants retained some SDH activity, the total lack of SDH activity would be lethal to *S. meliloti* cells. Indeed, previous studies in our lab indicated that transposon insertions that produce polar effects on other TCA cycle genes encoded in operons have apparently lethal effect (Dymov *et al.*, 2004). The results of this study have shown that while it is possible to mutate plasmid-borne *sdh* genes with transposons Tn*5* and Tn*5*-B20 (Figure 5.3), which can have polar effects, it is not possible to homogenotize the mutations into the genome using either plasmid incompatibility or *sacB* suicide vector recombination techniques. This finding is supported by previous observations for the genes of the *mdh-sucCDAB* operon (Dymov *et al.*, 2004). These experiments, directly targeting the *sdh* genes, supported the hypothesis that the complete absence of SDH activity, as would occur with Tn*5* insertion mutations, is lethal to *S. meliloti*, and perhaps to other aerobic species.

Three *sdh* mutants (two in *sdhC* and one in *sdhD* gene) were isolated through EZ::TN mutagenesis followed by recombination into the *S. meliloti* genome (Figure 5.3), and to the best of our knowledge this is the first report of genetically well-defined *sdh* mutants within the rhizobia. These mutants represent important

genetic tools in further understanding the regulation of genes encoding SDH and other TCA cycle enzymes.

To investigate the possible impact of polar effects on the *sdh* operon, our laboratory group has been trying to isolate a *sdh* mutant through random Tn*5*tac1 mutagenesis and screening for IPTG-dependent conditional mutants on minimal medium supplemented with succinate. Tn*5*tac1 is a derivative of Tn*5*, constructed to contain an outward-oriented *tac* promoter (P_{tac}) and the $lacI^q$ repressor (Chow and Berg, 1988) that can be induced to eliminate polar effects. This approach was used in our lab to isolate an *S. meliloti mdh*::Tn*5*tac1 mutant with reduced polar effects on the downstream *sucCDAB* genes and a conditional phenotype (Dymov *et al.*, 2004), however, no *sdh*::Tn*5*tac1 mutants have been isolated to date.

The growth phenotypes of the *sdh* mutants observed in this study were consistent with those reported in previous studies of *R. leguminosarum* (Finan *et al.*, 1981) and *S. meliloti* (Gardiol *et al.*, 1982) strains defective in SDH activity. The EZ::TN mutants were unable to grow on acetate, arabinose, glutamate, pyruvate and succinate. Interestingly, the same carbon sources (acetate, arabinose, glutamate) that demonstrated the highest level of *sdh::lacZ* expression (Figure 4.4) were unable to support growth of the *sdh* mutants. These results further support our previous hypothesis (presented in chapter 4) that the *sdh* operon is under metabolic regulation..

The mutants could not utilize any of the compounds tested that enter the TCA cycle and which cannot be cycled to the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PCK) without passing through SDH.

Pyruvate enters the TCA cycle via conversion by pyruvate carboxylase to oxaloacetate (OAA), and as OAA is the substrate of PCK, it would appear that either pyruvate carboxylase or PCK, or both, is not functioning under these conditions. Acetate enters via acetyl-CoA which is condensed with OAA via citrate synthase to form citrate. It was possible that these mutants could have grown on acetate if a functional glyoxylate shunt were to yield malate (Cronan and LaPorte, 1996), however, that is not occurring in these cells. Both glutamate and arabinose enter the cycle at 2-oxoglutarate dehydrogenase (Duncan and Fraenkel, 1979) and succinate enters just before SDH, and so it was not surprising that the mutants could not utilize these compounds. The growth, albeit poor, observed with fumarate, malate, would seem to be because these compounds could be converted to PEP via PCK. Thus it would seem that as long as *S. meliloti* cells are able to convert glycolytic (glucose) or gluconeogenic carbon sources into intermediary metabolites, growth is possible.

The enzyme activity of the SDH enzyme complex has been attributed to the two catabolic subunits SdhA and SdhB (Neidhardt *et al.*, 1996). This was further supported by Nakaruma *et al.* (1996), who reported detection of SDH activity in cytoplasmic but not in membrane fractions of *sdh⁻ E. coli* strains complemented with a plasmid borne copy of *sdhAB*. We observed similar findings with enzymes assays conducted on crude cell extracts and membrane preparations of Rm30168, Rm30169, and Rm30170. This further supports the notion SdhC and SdhD are involved in the anchoring of the enzyme complex while sdhA and SdhB alone are responsible for the actual oxidation of succinate. It is important to note that even in

terms of cytoplasmic SDH activity, the mutants expressed less than 40% of the *in vitro* SDH activity of the wild-type. A similar observation was reported with GFS18, an SDH defective *R. leguminosarum strain*, in which a 75% reduction in SDH activity was enough to confer a succinate negative growth phenotype.

As previously hypothesized, some SDH activity must be conserved to maintain a minimum flow through the TCA cycle. However the low level of SDH activity would most probably lead to an accumulation of metabolites and reducing power. Under those circumstances it would not be surprising to see an increase in the activity of other TCA cycle enzymes. The EZ::TN mutants demonstrated increased enzyme activity in all TCA cycle enzymes tested, including a ten-fold increase in MDH activity. An increase in MDH activity has also been reported in one of the previously isolated SDH *R. leguminosarum* mutant (Finan *et al.*, 1981) as well as in OGD mutants of *R. leguminosarum* (Walshaw *et al.*, 1997). From these results it can be concluded that increased metabolite concentration due to a partial block of the TCA cycle leads to the induction of other TCA cycle gene.

MDH is encoded by the *mdh* gene, which has been reported to be part of the *mdh-sucCDAB* operon (Dymov *et al.*, 2004). The *sucCD* and *sucAB* genes encode subunits of SCS and OGD, respectively. These genes have been shown to be co-transcribed as a single polycistronic mRNA from a promoter found upstream of *mdh* (Dymov *et al.*, 2004). As the genes encoding all of these enzymes are cotranscribed, an increase in MDH activity would also lead to higher OGD and SCS enzyme activities.

The TCA cycle has been shown to play a pivotal role in symbiotic N_2-fixing

bacteroids. This is supported by the observation of Nod^+ Fix^- symbiotic phenotypes of host plants when inoculated with mutants lacking TCA cycle enzymes: CIS (Kahn *et al.*, 1995), ICD (McDermott and Kahn, 1992), OGD (Duncan and Fraenkel, 1979), SCS (Walshaw *et al.*, 1997), MDH (Dymov *et al.*, 2004). In addition, malate and succinate are believed to be the carbon sources most likely provided to bacteroids (Vance and Heichel, 1991). Therefore it is not surprising that mutants defective in SDH would result in nodules unable to fix N_2. Fix^- phenotype were observed with all three *sdh* mutants. The same symbiotic phenotype was also reported by the very few studies involving SDH in rhizobia (Finan *et al.*, 1981; Gardiol *et al.*, 1982). Further demonstrating the requirement of a complete TCA cycle including SDH in bacteroids.

The complementing cosmid, pFD71, carrying the complete *sdh* operon, could partially but not completely restore the wild-type symbiotic-phenotype in the *sdh* mutants. It is believed that loss of the plasmid under non-selective conditions along with plasmid instability within nodules could account for difference in SDW observed between the wild-type and complemented mutants. Driscoll and Finan (1997) have demonstrated the high rate of plasmid loss in bacteroids under non-selective conditions. This was further supported by Dymov *et al.* (2004) observation that only 13% of colonies recovered from nodules generated from a complemented *mdh* mutant retained the complementing plasmid.

Chapter 6. Conclusions and general discussion

Reduced sensitivity to the plant signal, flavonoids, has been postulated as one of the possible mechanisms involved in *nod* gene repression under low RZT (Zhang and Smith 1996b). To overcome this obstacle, ten *B. japonicum* mutants with altered *nod* gene induction characteristics were isolated through UV mutagenesis. Our hypothesis was that genistein-independent strains could be used to circumvent the delays seen in nodulation under suboptimal temperatures. While the mutants initially appeared to have *nod* genes that were being constitutively expressed (expressed in the absence of genistein induction), they were determined to have enhanced sensitivity to genistein with respect to *nod* gene induction instead. The mutants' ultrasensitivity to the plant signal did, however, translate into increased LCO production compared to the wild-type under the same conditions. This increase in LCO production was also observed when the mutants where compared to *B. japonicum* strain 532C a widely used inoculum in Canadian agriculture (Zhang *et al.*, 2002c).

The fact that the mutants were not constitutive *nod* gene expression strains could actually have been beneficial. Since previous findings demonstrated that over-expression of the *nod* genes in bacteroids resulted in disruption of N_2-fixation activity (Knight *et al*, 1986; Schlaman *et al*, 1991). It is not uncommon for bacteria to regulate biosynthetic operons via a feedback mechanism, reducing or even preventing unwanted productions of these products (Goldberger, 1974). In this regard, Nod factor or parts of the Nod factor have been shown to repress *nod* gene

expression in *B. japonicum* (Loh and Stacey, 2001). In addition, the *nod* genes are believed to be regulated in a population density-dependent fashion through the action of Bradyoxetin. This quorum sensing regulation has been proposed to be responsible for the repression of *nod* genes in nodules. The high cell-to-volume ratio in symbiosomes would replicate an environment of high population density (Loh *et al.*,2001). Determination of the symbiotic phenotype revealed that three of the ten mutants had indeed a Fix$^-$ phenotype. It is interesting to note that one of these mutants (Bj30056) was the only mutant to show any *nod* expression in the absence of genistein (Figure 3.2).

However, since the mutant strains were not true constitutive *nod* gene mutants but rather ultrasensitive to genistein could help explain why we observed slight increases in SDW on soybean plants inoculated with four of the mutant strains. These increases were further demonstrated over a two year field experiment in which the mutants recorded a greater number of nodules, nodule dry weight, shoot nitrogen yield, and total nitrogen fixation then wild-type (Zhang *et al.*, 2002a,b). Because of the nature of the mutation, it is highly possible that more than one mutation could be responsible for the phenotypes observed. However, the mutant *B. japonicum* strains generated in this investigation remain a useful tool for further studies on the *nod* gene regulation as well as prime candidates for use as commercial inoculums.

The importance of the TCA cycle in symbiotic N_2-fixation is well established (Dunn, 1998). Prior to this study very little was known in respect to SDH in rhizobia, the information that was available was mostly based on enzymatic

studies or poorly genetically defined mutants (Gardiol, *et al.*, 1982; Finan *et al.*, 1981). Using both transcriptional fusions and SDH defective mutants, this study was able to demonstrate that the *sdh* operon, as well as possibly other TCA cycle enzymes, are under metabolic regulation.

Previous attempts to isolate well-defined *sdh* mutants through transposon mutagenesis were unfruitful (Finan, *et al.*, 1981, Dymov, *et al.*, 2004). The results of this study have shown that while it is possible to mutate plasmid-borne *sdh* genes with transposons, which can have polar effects, it is not possible to homogenotize the mutations into the genome using either plasmid incompatibility or *sacB* suicide vector recombination techniques. This led to the hypothesis that the complete absence of SDH activity, as would occur with Tn*5* insertion mutations, is lethal to *S. meliloti*, and perhaps to other aerobic species.

Three *sdh* mutants (two in *sdhC* and one in *sdhD* gene) were isolated through EZ::TN mutagenesis followed by recombination into the *S. meliloti* genome (Figure 5.3), and to the best of our knowledge this is the first report of genetically well-defined *sdh* mutants within the rhizobia. We therefore believe that EZ::TN will become a useful tool in isolating more mutants in genes that are particularly susceptible of generating lethal polar effects on downstream genes.

The genes of the *mdh-sucCDAB* operon would appear to be a prime candidates for the use of this technique. An *mdh* mutant was recently isolated in *S. meliloti* using Tn*5*tac1 (Dymov *et al.*, 2004). Tn*5*tac1 ensures the expression of downstream genes by carrying an outward-oriented *tac* promoter (P_{lac}) and the $lacI^q$ repressor (Chow and Berg, 1988), however this promoter impacts expression of the

downstream genes, making interpretations difficult. Hopefully EZ::TN mutagenesis will prove useful in isolating mutants that have no polar effects and thus no impact on downstream expression.

As previously hypothesized, some SDH activity must be conserved to maintain a minimum flow through the TCA cycle. However the low level of SDH activity would most probably lead to an accumulation of metabolites and reducing power. This was demonstrated in enzyme assays performed on the *sdh* mutants, that showed increased expression of TCA cycle enzymes excluding SDH. The question remains however, that if some SDH activity is present in the mutants, why could they not utilize succinate as a sole carbon source? Future experiments will have to be designed in order to get a better understanding of carbon metabolism in *S. meliloti* free-living cells and bacteroids, and the *sdh* mutants isolated in this study will be invaluable tools for these investigations.

Sequence analysis revealed that the four subunits that encode the SDH enzyme complex are found on the chromosome in an operon with a *sdhCDAB* gene order. Using RT-PCR analysis the *sdh* genes were shown to be co-transcribed as a single polycistronic mRNA from a single promoter upstream of *sdhC*. This is different from the organization of the *E. coli* operon, in which the *sdh* operon also includes genes encoding subunits of OGD and SCS (Cunningham and Guest, 1998). The difference in gene organization could be reflective of the contrast observed in the regulation of the *sdh* genes between these organisms. In rhizobia, SDH is most likely associated with a TCA cycle that operates under aerobic and microaerophilic (during symbiosis) conditions, whereas in *E. coli*, SDH has been shown to be

repressed under anaerobic conditions (Park *et al.*, 1995).

The SDH activity of the SDH enzyme complex has been shown to be associated with the catabolic subunits alone (Neidhardt *et al.*, 1996). Enzyme assays performed on *sdh* mutant crude extracts and membrane fractions supported this notion. The enzyme assays also demonstrated that SdhC and SdhD are required for proper anchoring of the catabolic subunits to the membrane.

The Nod^+ Fix^- phenotype observed on plants inoculated with the *sdh* mutants indicated that SDH is required for the establishment of symbiotic N_2-fixation. The N_2-fixation ability of the mutants was partially restored with the introduction of a plasmid borne copy of the complete *sdh* operon

The work conducted in this investigation was carried out in the hopes of acquiring knowledge that could be used to alleviate the inhibition of nodulation of legumes by rhizobia observed in temperate climates (Turgeon and Bauer 1982; Zhang and Smith, 1994). The information that was gathered on the regulation of both *nod* and *sdh* genes in this study will hopefully lead to such outcomes. We believe that the mutants isolated in *B. japonicum* and *S. meliloti* represent excellent tools to further our understanding of the highly complicated processes of symbiotic N_2-fixation.

Connecting text

The work performed in Appendix 1 was designed to study the potential impact of plant-growth-promoting bacteria (PGPB) on the growth of soybean plants. Three non-*Bradyrhizobium* endophytic bacteria (NEB) were isolated and identified. The majority of the experimental work, accompanying analysis, and writing of this chapter was conducted by myself, with the exceptions cited in contributions of authors (p.xx). Dr. Brian Driscoll provided critical reading of the chapter and suggested ways in which to improve the work. The published manuscript was originally written by myself and subsequently edited and revised by Dr. Brian Driscoll, with contributions from the other authors.

Appendix 1. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules

This appendix was adapted from the following:

Bai, Y., D'Aoust, F., Smith, D.L., and Driscoll, B.T. 2002. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. Can. J. Microbiol. **48**:230-238.

SUMMARY

Endophytic bacteria reside within plant tissues and have often been found to promote plant growth. Fourteen strains of putative endophytic bacteria, not including endosymbiotic *Bradyrhizobium* strains, were isolated from surface-sterilized soybean (*Glycine max.* (L.) Merr.) root nodules. These isolates were designated as non-*Bradyrhizobium* endophytic bacteria (NEB). Three isolates (NEB4, NEB5, and NEB17) were found to increase soybean weight when plants were co-inoculated with one of the isolates and *Bradyrhizobium japonicum* under nitrogen-free conditions, compared with plants inoculated with *B. japonicum* alone. In the absence of *B. japonicum*, these isolates neither nodulated soybean, nor did they affect soybean growth. All three isolates were Gram-positive spore-forming rods. While Biolog tests indicated that the three isolates belonged to the genus *Bacillus*, it was not possible to determine the species. Phylogenetic analysis of 16S

rRNA gene hypervariant region sequences demonstrated that both NEB4 and NEB5 are *Bacillus subtilis* strains, and that NEB17 is a *Bacillus thuringiensis* strain.

INTRODUCTION

Plants are constantly involved in interactions with a wide range of bacteria. These plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes), and the inside of plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant, and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots, and seeds of various plant species (Kobayashi and Palumbo, 2000). Depending on their effect on the host plant, endophytic bacteria can be categorized into three groups: plant-growth promoting, plant-growth inhibiting, and plant-growth neutral (Sturz *et al.*, 2000).

Bacterial strains that have beneficial effects on plant health are referred to as beneficial plant-associated bacteria, plant-growth-promoting bacteria (PGPB), or plant-growth-promoting rhizobacteria (PGPR; Andrews and Harris 2000). PGPB can promote plant growth directly or indirectly via biocontrol of host plant diseases, production of phytohormones, or improvement of plant nutritional status (Glick, 1995). Rhizobia are perhaps the best known beneficial plant-associated bacteria because of the importance of the nitrogen fixation that occurs during the Rhizobium–legume symbiosis. The co-inoculation of other PGPB with rhizobia is becoming a practical method in the development of sustainable agriculture due to

the yield increases seen compared with inoculation with rhizobia alone. PGPB that have been tested as co-inoculants with rhizobia include strains of the following well-known rhizobacteria: *Azospirillum* (Yahalom *et al.*, 1987), *Azotobacter* (Burns *et al.*, 1981), *Bacillus* (Srinivasan *et al.*, 1996), *Pseudomonas* (Chanway *et al.*, 1989), *Serratia* (Chanway *et al.*, 1989; Zhang *et al.*, 1997), and *Streptomyces* (Li and Alexander, 1988).

Endophytic bacteria have been isolated from legume plants such as alfalfa (Gagne *et al.*, 1987), clover (Sturz *et al.*, 1997), pea (Elvira-Recuenco and van Vuurde, 2000), and soybean (Oehrle *et al.*, 2000). Bacteria of several genera have been isolated from legume tissues including *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavimonas*, *Pseudomonas*, and *Sphingomonas* (Gagne *et al.*, 1987; Sturz *et al.*, 1997; Elvira-Recuenco and van Vuurde, 2000; Oehrle *et al.*, 2000). Sturz *et al.* (1997) reported the isolation of 15 nonrhizobial species from clover root nodules, eight of which were found only in root nodule tissues.

Endophytic PGPB have the potential to be used as agricultural inoculants (Hallmann *et al.*, 1997; Sturz *et al.*, 2000). PGPB have been isolated by screening the rhizosphere, phyllosphere, and the tissues of plants showing particularly vigorous growth in the field. This approach was used to isolate the effective plant-growth-promoting *Bacillus megaterium* strain B153-2-2 from a field-grown soybean plant (Liu and Sinclair, 1993). Our goal was to use a similar approach to isolate endophytic PGPB from the root nodules of particularly vigorous field-grown soybean plants

RESULTS

Isolation of endophytic bacteria from surface-sterilized nodules

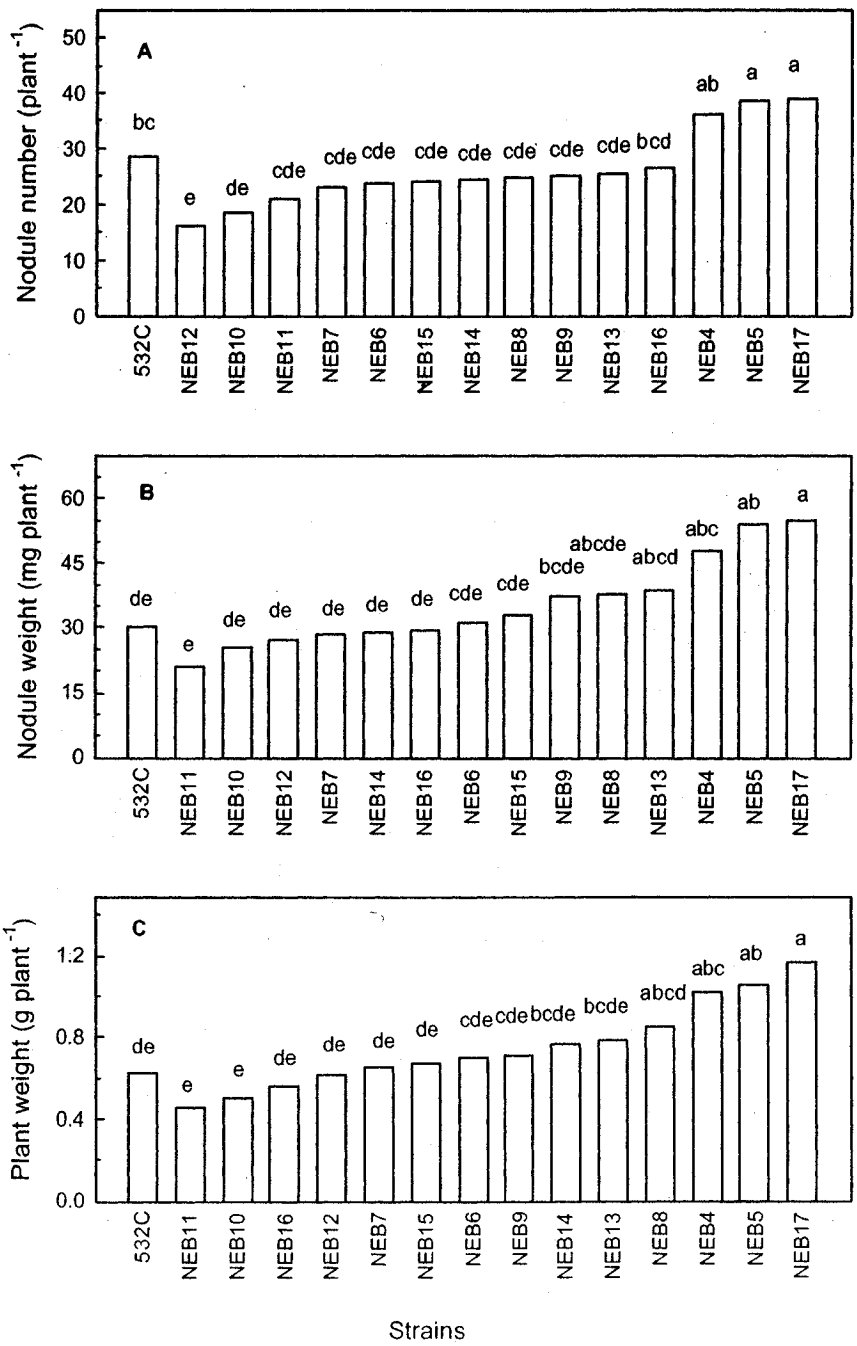
We wished to isolate non-*Bradyrhizobium* bacteria from within soybean root nodules. On August 14, 1998, twenty vigorous soybean (*Glycine max* (L.) Merr.) seedlings at the R3 stage (Fehr *et al.*, 1971) were selected from five fields at the A.E. Lods Agronomy Research Centre, Macdonald Campus, McGill University. The fields had been sown with soybean cultivars OAC Bayfield and OAC Maple Glen and inoculated with *B. japonicum* 532C as described (Dashti *et al.*, 1997). Twenty nodules, four from each of the five fields, were placed into separate sterile Eppendorf tubes with 1 ml of sterile H_2O. To confirm nodule surface sterility, immediately following surface sterilization, the nodules were crushed aseptically, nodule contents were streaked onto YEM plates, and the plates were incubated at 28°C. Non-*Bradyrhizobium* colonies were chosen on the basis of colony morphology and growth rate. After four days, non-*Bradyrhizobium* colonies were picked and then were purified by single-colony streaking on three successive King's Medium B plates. A total of 14 strains with distinct colony morphologies (three strains from one nodule, two each from three nodules, and one each from five nodules) were kept for further study. Isolates were only retained from nodules that were confirmed to have been surface sterilized. The putative nodule endophyte strains were designated as NEB.

Effects of the NEB strains on soybean plant growth

Growth pouch experiments were arranged following a completely randomized split plot design with three replicates per inoculation treatment (Mead *et al.*, 1993). Soybean seedlings were co-inoculated with *B. japonicum* 532C and each of the 14 distinct NEB isolates. Plant weight, nodule number, and nodule weight were determined 55 days after inoculation (Figure A.1). When analysis of variance indicated differences among means, comparisons among the treatment means were conducted with an ANOVA protected least significance difference (LSD) test (Steel and Torrie, 1980). Nodule number and plant dry weight were determined and the nitrogen content of dried plants (shoot plus root) was determined using the Kjeldahl method (Kjeltec system, with Digestion System 20, and a 1002 Distilling Unit, Tecator AB, Hoganas, Sweden), as previously described (Bremner, 1965). Control values for plant dry weight and nitrogen content were 864 ± 68 and 11.1 ± 1.4 mg/plant, respectively (mean ± SD, $n = 6$).

While the majority of the isolates had no significant effects on soybean growth and development, three (NEB4, NEB5, and NEB17) appeared to have positive effects. Plants co-inoculated with these strains had significantly higher nodule and plant weights, both NEB5 and NEB17 seemed to increase nodule number per plant. These strains also had positive effects on soybean growth when the root zone temperature was lowered. Isolates NEB10, NEB11, and NEB12 seemed to be the poorest performers overall, with some significant decreases in plant weight and nodule number compared with the control. The remaining isolates had no significant effects on soybean growth or nodulation. All further

Figure A.1. Effects of NEB strains on soybean plants co-inoculated with *B. japonicum*. Plants were cultured in growth pouches with N-free Hoagland's solution, harvested 55 days after inoculation, and then nodule number (A), nodule weight (B), and plant weight (C) were determined. Control plants (532C) were inoculated with *B. japonicum* 532C alone, all other plants were inoculated with *B. japonicum* 532C plus one of the NEB strains, as indicated. The bars represent the mean values (n = 6), and the letters above each bar indicate the differences at the P = 0.05 level.


A
Nodule number (plant -1)
0
10
20
30
40
50
bc
e
de
cde
cde
cde
cde
cde
cde
cde
cde
bcd
ab
a
a
532C
NEB12
NEB10
NEB11
NEB7
NEB6
NEB15
NEB14
NEB8
NEB9
NEB13
NEB16
NEB4
NEB5
NEB17
B
Nodule weight (mg plant -1)
0
15
30
45
60
de
e
de
de
de
de
de
cde
cde
bcde
abcde
abcd
abc
ab
a
532C
NEB11
NEB10
NEB12
NEB7
NEB14
NEB16
NEB6
NEB15
NEB9
NEB8
NEB13
NEB4
NEB5
NEB17
C
Plant weight (g plant -1)
0.0
0.4
0.8
1.2
de
e
e
de
de
de
de
cde
cde
bcde
bcde
abcd
abc
ab
a
532C
NEB11
NEB10
NEB16
NEB12
NEB7
NEB15
NEB6
NEB9
NEB14
NEB13
NEB8
NEB4
NEB5
NEB17
Strains

experiments were limited to the soybean-growth-promoting strains NEB4, NEB5, and NEB17.

There was no evidence that the positive soybean-growth effects of NEB4, NEB5, and NEB17 were as a result of supplying the plants with fixed nitrogen. The strains were each inoculated onto soybean seedlings, as above, but in the absence of *B. japonicum* 532C. None of these strains were able to form root nodules with soybean. The plants appeared chlorotic and stunted, similar to uninoculated control plants. Neither the plant weights nor their nitrogen contents were significantly different from those of uninoculated control plants.

Phenotypic characterization of the NEB strains

Distinct colony morphologies were observed for NEB4, NEB5, and NEB17 on King's Medium B plates. NEB4 and NEB5 colonies both had slimy capsules, and produced red, water-soluble, pigments. NEB17 colonies had a waxy appearance, with no pigment. The cultures were tested for the presence of spores using the Schaeffer–Fulton staining method and for Gram reaction. All three strains were determined to be Gram-positive spore-forming rods. As all three strains were found to be Gram positive, they were assayed for carbon utilization using Biolog GP Microplates (Biolog Inc., Hayward, Calif.), following the manufacturer's instructions. *Staphylococcus aureus* and *Bacillus cereus* were used as controls.

The NEB strains could not be identified at the species level using the Biolog system because of a very high percentage of false-positive results. This result was anticipated, however, as spore-forming bacteria such as *Bacillus* species frequently

yield false-positives in Biolog tests. This phenomenon is discussed in the Biolog technical literature and has been observed by others (Baillie *et al.*, 1995). Despite numerous attempts, the SIM values for the NEB strains and the *B. cereus* control (0.315) were below the threshold of 0.5 acceptable for species identification. The SIM value for the (non-spore-forming) *S. aureus* control was, however, 0.563. The Biolog database matches with the highest SIM values were to *B. subtilis* for both NEB4 (0.242) and NEB5 (0.426). For NEB17, the best matches were to *B. mycoides* (0.483), *B. cereus* (0.417), and *B. thuringiensis* (0.417). Therefore, while these tests indicated that the NEB strains were *Bacillus* species, they did not provide identifications at the species level.

Phylogenetic analysis

The complete 1.6-kb 16S rDNA region was amplified using the universal bacterial 16S rDNA primers 27f and 1492r. PCR products were ligated into the vector pGEM-T Easy and ligation products were transformed into $CaCl_2$-competent *E. coli* DH5α cells using the materials and protocols supplied with the vector (Promega Inc., Madison, Wis.). Plasmid DNA was isolated from positive clones and purified prior to sequencing, as described in chapter 2. DNA sequencing was done using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Mississauga, Ont.) and standard T7 and SP6 promoter sequencing primers (Gibco-BRL). Sequencing reactions were run on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems). Nucleotide sequences were compiled using Sequencher v. 3.0 (Gene Codes Corporation, Inc., Ann Arbor,

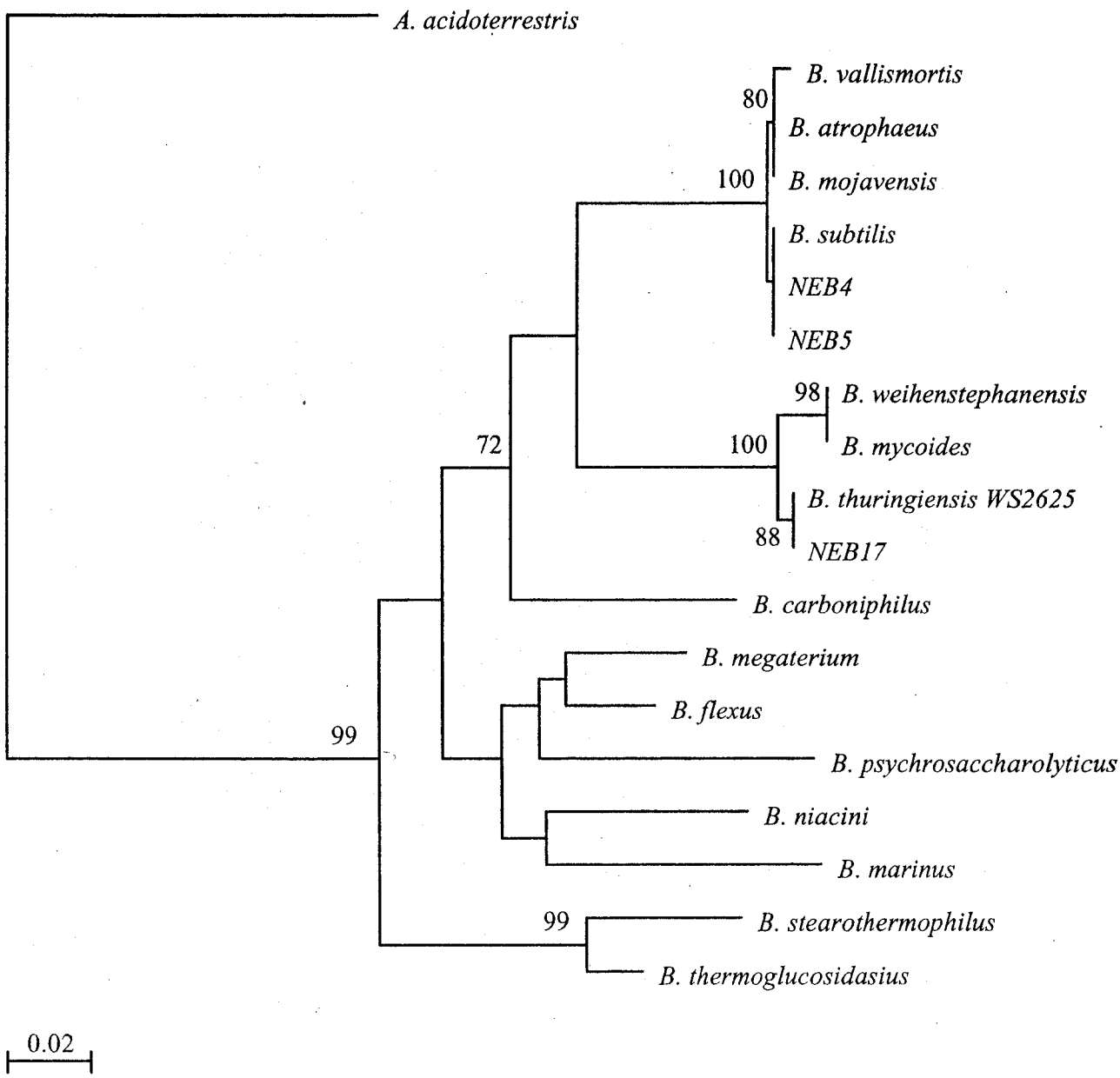
Mich.).

The NEB4 and NEB5 sequences were identical to each other. BLAST comparisons, done to verify that the clones contained 16S rDNA, revealed that the NEB17 sequences had very high homology to the 5' and 3' ends of the *B. thuringiensis* WS2625 16S rRNA gene and that the NEB4 and NEB5 sequences had very high homology to the 5' and 3' ends of *B. subtilis* 16S rRNA genes.

As all indications were that the three NEB strains were *Bacillus* species, we utilized PCR primers designed to amplify the hypervariant (HV) region of *Bacillus* 16S rDNA (Goto *et al.*, 2000). The PCR amplifications yielded single PCR products of the expected size, approximately 350 bp, for each strain. The PCR products were cloned and nucleotide sequences were generated for both strands. The NEB4 and NEB5 HV sequences (275 nucleotides) were identical and were identical to those of 13 *B. subtilis* strains. The NEB17 HV sequence (277 nucleotides) was identical to *B. thuringiensis* strain WS2625. The NEB4 (275 nucleotides), NEB5 (275 nucleotides), and NEB17 (277 nucleotides) 16S rRNA gene HV sequences were deposited in GenBank under accession numbers AF406704, AF406705, and AF406706 respectively.

A neighbor-joining dendrogram was generated using the HV sequences from the NEB strains and representative *Bacillus* sequences from GenBank (Figure A.2). As expected, NEB4 and NEB5 clustered with *B. subtilis* and NEB17 clustered with *B. thuringiensis* WS2625. The separation of the NEB4 – NEB5 – *B. subtilis* cluster from the *B. vallismortis* – *B. mojavensis* – *B. atrophaeus* cluster was supported by a bootstrap value of 100%. The separation of the NEB17 – *B. thuringiensis* WS2625

Figure A.2. Phylogenetic relationships between NEB4, NEB5, NEB17, and representative Bacillus species based on 16S rDNA HV sequences. The dendrogram was generated by the neighbor-joining method, with Kimura distances, and is rooted to the out-group *A. acidoterrestris*. Nodes with greater than 70% bootstrap support (1000 replications) are indicated. The bar represents 0.02 nucleotide substitutions per site. Accession numbers are reported in the chapter 2.


A. acidoterrestris
B. vallismortis
80
B. atrophaeus
100
B. mojavensis
B. subtilis
NEB4
NEB5
98
B. weihenstephanensis
72
100
B. mycoides
B. thuringiensis WS2625
88
NEB17
B. carboniphilus
B. megaterium
B. flexus
99
B. psychrosaccharolyticus
B. niacini
B. marinus
99
B. stearothermophilus
B. thermoglucosidasius
0.02

cluster from the *B. weihenstephanensis* – *B. mycoides* cluster also had 100% bootstrap support. The same tree topology and high bootstrap values were achieved using Tamura–Nei distances (results not shown). The phylogenetic relationships between species related to the NEB strains as well as those between HV sequences of other *Bacillus* species, particularly those from the *B. megaterium* and *B. stearothermophilus* clusters, were reconstructed as previously reported (Goto *et al.*, 2000).

DISCUSSION

Fifty-three percent of the surface-sterilized soybean nodules tested carried non-*B. japonicum* bacteria and several carried more than one morphologically distinct strain. Endophytic bacteria have previously been isolated from legume root nodules. Sturz *et al.* (1997) characterized 15 bacterial species from red clover nodules and estimated endophyte population densities to be in the range of 10^4 viable bacteria per gram fresh nodule. In plant tissue in general, endophytic bacterial populations have been reported between 10^2 and 10^4 viable bacteria per gram (Kobayashi and Palumbo, 2000).

There is much debate as to how to define an endophyte (reviewed in Kobayashi and Palumbo, 2000). Hallman *et al.* (1997) suggested that bacteria that are isolated from surface-sterilized plant tissues, and that do no apparent harm to the plant, could be considered endophytes. Other definitions suggest that it is necessary to demonstrate that the bacterial colonization is of internal plant tissues (Kobayashi

and Palumbo, 2000). While our results indicate that NEB4, NEB5, and NEB17 are PGPB, we have not pursued definitive tests that could prove whether these strains are endophytes or epiphytes. As the strains were isolated from surface-sterilized root nodules, we labeled them as putative endophytes; however, we recognize that they may not prove to be colonizing internal plant tissues. In future studies it would be interesting to attempt to re-isolate the NEB strains from surface-sterilized root nodules of plants co-inoculated with *B. japonicum*. This type of study, and perhaps microscopic examination of nodule tissue, would aid in determining whether these strains should be defined as endophytes. Until that work is done, the strains should simply be considered as PGPB. One reason for this uncertainty is that although the root nodules used in our experiments appeared to be surface sterilized, the three strains examined in detail (NEB4, NEB5, and NEB17) were all endospore formers. Bacterial endospores bound to the surface of the nodules may have survived the surface sterilization.

Of the 14 isolates, only NEB4, NEB5, and NEB17 improved soybean nodulation and plant weight when co-inoculated with *B. japonicum* 532C under N-free culture conditions, compared with plants inoculated with *B. japonicum* alone. These three strains were incapable of either nodule formation or nitrogen fixation with soybean, nor did they enhance plant growth when inoculated without *B. japonicum*. None of the strains appeared to be able to fix nitrogen for growth in N-free media. Whether these three NEB strains could promote soybean growth under conditions with full or limited N supply needs to be determined. Further experiments to determine whether these strains can colonize other plant tissues or

plant species would be of interest. In addition it may be useful to try to determine whether the increased plant weight was due to increased nodule weight or vice versa.

Endophytic bacteria isolated from red clover have also been reported to promote host plant growth as well as nodulation more often when co-inoculated with *Rhizobium leguminosarum* as opposed to when applied alone (Sturz *et al.*, 1997). We have previously described PGPR strains, *Serratia proteamaculans* 1-102 and *Serratia liquefaciens* 2-68, which enhance soybean nodulation and improve both plant growth and grain yield (Zhang *et al.*, 1996b; Dashti *et al.*, 1997).

Bacillus species have been shown to have positive soybean-growth effects. *Bacillus megaterium* B153-2-2 appears to enhance plant growth and nodulation by *B. japonicum* by inhibiting the growth of the phytopathogen *Rhizoctonia* on the host plant (Liu and Sinclair, 1993). In addition to legumes, *Bacillus* endophytes have been isolated from many different plant tissues, such as cotton (Misaghi and Donndelinger, 1990), potato (Sturz and Christie, 1995), citrus (Araujo *et al.*, 2001), oak, maple, cauliflower, grape, corn, and sunflower (Kobayashi and Palumbo, 2000).

Colony morphology, carbon and nitrogen use in Ashbey's media, and Biolog tests indicated that three NEB isolates of interest were *Bacillus* strains. The Biolog tests did not yield conclusive species identifications and the difficulty of identifying *Bacillus* species (*B. thuringiensis* in particular) has been documented previously (Baillie *et al.*, 1995). The oxidation of reserves by germinating spores is thought to be at least one reason why these species frequently yield false-positive results in

Biolog tests.

Since preliminary analysis of 16S rDNA sequences also indicated that the NEB strains were *Bacillus* species, it was possible to perform phylogenetic analysis using the approximately 275 nucleotides from the HV region of the 16S rDNA. Goto *et al.* (2000) demonstrated that many *Bacillus* strains may be reliably classified to the species level on the basis of the sequences of a minimum of 219 nucleotides from the HV region and validated this for numerous strains in the *B. subtilis* cluster (*B. subtilis – B. mojavensis – B. atrophaeus – B. vallismortis*).

The situation with respect to the identification of *B. thuringiensis* strains is more complex. *Bacillus thuringiensis* falls into the "*B. cereus* group" of species, including *B. cereus*, *B. mycoides*, *B. anthracis*, and *B. weihenstephanensis.* Numerous phenotypic and genotypic characterization methods have been applied to this group, and strains of one species are often found to be closer taxonomically to another species than to their own type strain (Bourque *et al.*, 1995; Lechner *et al.*, 1998; Daffonchio *et al.*, 2000). Indeed, it has been suggested that *B. anthracis*, *B. cereus*, and *B. thuringiensis* may belong to one species (Helgason *et al.*, 2000). Although no system for classifying these species has been agreed upon, we found that the results of sequencing the HV region strongly suggested that NEB17 is a *B. thuringiensis* strain, as this sequence was identical to *B. thuringiensis* WS2625 and to nothing else. Sequences with lower homology to NEB17 were from various species within the "*B. cereus*" group.

Phylogenetic analysis of the HV sequences of the NEB trains and key *Bacillus* species (Figure A.2) demonstrated that both NEB4 and NEB5 are strains of

B. subtilis, and NEB17 is a *B. thuringiensis* strain related to *B. thuringiensis* WS2625. The clusters into which the NEB HV sequences fit were the same as those reported by Goto *et al.* (2000) and had high bootstrap support. In addition, the sequences obtained for all three NEB strains were 100% identical to previously characterized *Bacillus* strains. Therefore, we have designated the nodule isolates as *B. subtilis* NEB4, *B. subtilis* NEB5, and *B. thuringiensis* NEB17 and propose that these strains may be useful in agriculture, as co-inoculants with *B. japonicum*. As all three strains form endospores, they should be readily adaptable to commercial formulation and application to field crops (Liu and Sinclair, 1993).

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Brian Driscoll PHONE: 398-7887

DEPARTMENT: Natural Resource Sciences FAX: 398-7990

ADDRESS: 21,111 Lakeshore Rd, Ste-Anne-de-Bellevue E-MAIL: driscoll@nrs.mcgill.ca

PROJECT TITLE: Sinorhizobium meliloti TCA cycle genetics

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Brian Driscoll Phone No: work: 398-7887 home: 428-5244

Name: _____ Phone No: work: _____ home: _____

3. FUNDING SOURCE OR AGENCY (specify): NSERC

Grant No.: RGPIN183975-01 Beginning date: April 2001 End date: March 2006

4. Indicate if this is

☑ Renewal: procedures previously approved without alterations.

Approval End Date: grant extended by one year automatically

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency: _____ Approval End Date: _____

☐ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☑ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ (SIGNATURE) date: 21 (day) January (month) 2005 (year)

Approved by Environmental Health & Safety: _____ (SIGNATURE) date: 25 (day) 01 (month) 05 (year)

Expiry: 31 (day) 03 (month) 06 (year)

s defined in the "McGill Laboratory Biosafety Manual"

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes indicate training date.
Jean-Francois Lauzon	Natural Resource Scienc	Graduate student	
David Meek	Natural Resource Scienc	Technician	
Lynda Stewart	Natural Resource Scienc	Graduate student	
Frederic D'Aoust	Natural Resource Scienc	Graduate student	

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

Bacteria from the genus Sinorhizobium, as well as other related bacteria (Rhizobia), are nitrogen-fixing symbionts of legume plants. These bacteria were previously isolated from healthy plants and none are known pathogens. We will also utilize standard (non-pathogenic) strains of Escherichia coli and bacteriophage specific to Sinorhizobium meliloti and E. coli for molecular genetic and cloning experiments.

ii) the procedures involving biohazards

DNA extraction and cloning from Rhizobia. These strains will also be grown with plant hosts in plant growth chambers to test symbiotic nitrogen fixation. We will be growing these strains and bacteriophage in liquid culture and on agar plates. Most of the work will involve molecular biological techniques using DNA maintained in plasmids in an E. coli background. We will be using standard bacterial genetics protocols such as conjugation, transformation and transduction.

iii) the protocol for decontaminating spills

Spills will be mopped up with paper towels, surfaces will be sterilized using ethanol or dettol, and contaminated material will be autoclaved (contaminated paper in biohazard bags) and discarded.

. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could ıcrease the hazards?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

Use of pipette guns rather than glass pipettes, regular maintenance of pipette guns, use of disposable tips, use of capped tubes, training in asceptic technique using bunsen burner. Any pathogen-containing material can be manipulated in a vertical flow hood located in Dr. Niven's lab (MS3-050).

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

No

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

Np

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Macdonald Stewart	MS3-050	Canadian cabinets	BM6 2A	7079	Sept 2004

McGill University
Environmental Safety

THIS IS TO CERTIFY THAT

Frederic D'Aoust

HAS SUCCESSFULLY COMPLETED A BASIC

LABORATORY COURSE IN

RADIATION SAFETY

December 2003

Joseph Vincelli

Radiation Safety Officer
J. Vincelli

Manager, Environmental Safety
W. Wood, ROH

August 11, 2005

To Whom It May Concern:

Mr. Frederic D'Aoust has requested permission to reprint and use an article published in our journal, *Molecular Plant-Microbe Interactions,* in his Ph.D. thesis at McGill University: Isolation and genetic characterization of nod and sdh operons in Rhizospheric bacteria *Bradyrhizobium japonicum* and *Sinorhizobium meliloti.*

Permission is hereby granted for him to include the following article, limited to his thesis and provided the source is properly credited:

> Ip, H., D'Aoust, F., Begum, A.A., Zhang, H., Smith, D.L., Driscoll, B.T., and Charles, T.C. 2001. *Bradyrhizobium japonicum* mutants with enhanced sensitivity to genistein resulting in altered nod gene regulation. Mol. Plant Microbe Interact. 14:1404-1410.

Sincerely,

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